VOLUME XXXIII NOVEMBER, 1954 NUMBER 11

# The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

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# The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation. Publication Office, 622 West 168th St., New York 32, N. Y.

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Acceptance for mailing at special rate of postage provided for in paragraph (d-2), section 34.40, P.L. & R. of 1948, authorized February 13, 1950

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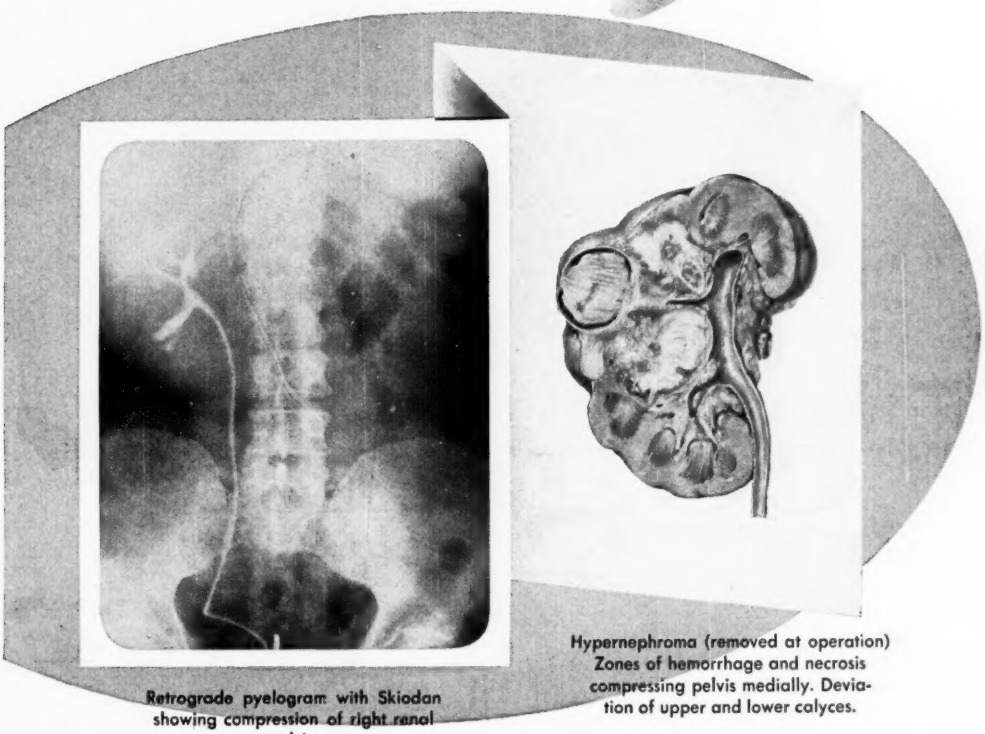

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**References**
(1) Kirby, W. M. M.; Waddington, W. S., & Doornink, G. M.: Antibiotics Annual, 1953-1954, New York, Medical Encyclopedia, Inc., 1953, p. 285. (2) Finland, M., & Haight, T. H.: *Arch. Int. Med.* **91**: 143, 1953.

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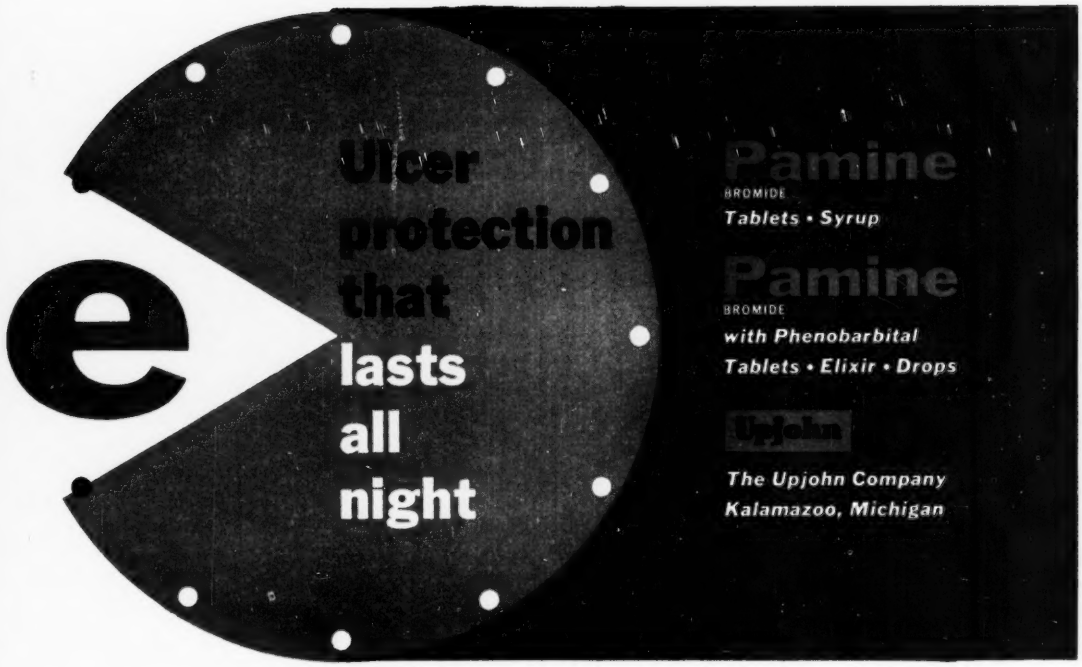


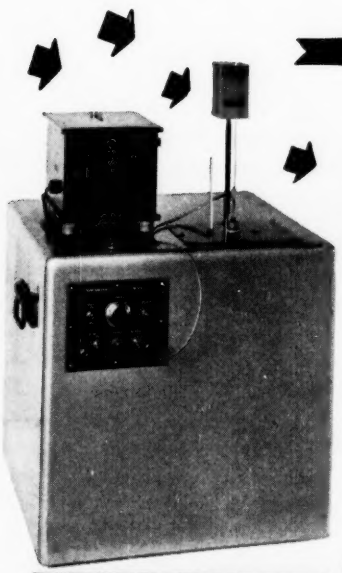

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# THE MIDDLEBROOK–DUBOS HEMAGGLUTINATION TEST FOR TUBERCULOSIS IN CHILDREN AND ADULTS[1]

By ANTON JOSIUKAS, T. E. ROY, AND GLADYS BOYD

*(From The Department of Pediatrics, University of Toronto, The Research Institute of The Hospital for Sick Children, and The Department of Bacteriology, University of Toronto, Toronto, Canada)*

(Submitted for publication May 10, 1954; accepted June 23, 1954)

Middlebrook and Dubos (1) described a hemagglutination reaction designed to detect serum antibodies to certain components of M. tuberculosis. Early reports suggested that this test might have valuable clinical applications both in the diagnosis of tuberculosis and in evaluating the activity of the disease (2–7). Subsequent reports on its clinical usefulness have been less encouraging (8–16). Many modifications of the test have been devised in an attempt to make it more specific. These have included more thorough absorption of sera with normal sheep erythrocytes to remove heterophile antibodies (17), the substitution of human Group O cells for sheep erythrocytes (18–21), the use of fowl erythrocytes (22), and the use of collodion particles (23). Middlebrook (24) introduced a hemolytic modification which has received considerable study (25–33). A slide agglutination test has been used (34). Different products of the tubercle bacillus have been used (1, 5, 15, 20, 23, 29, 33, 35, 36) in attempts to make the test more specific, and modifications of the method of adsorbing antigens on the erythrocytes have included the use of tannic acid (37–39), inulin or trypsin (38), and treatment of the cells with Forssman antibody (30). In an effort to explain false negative reactions, "incomplete" or "blocking" antibodies have been looked for by the use of such diluents as pooled serum, 5 per cent bovine albumin, glycine solution, or polyvinyl pyrrolidone (30), and by the use of the Coombs antiglobulin technique (26, 40). None of the modifications appears to have improved the test, and the consensus is that it is not sufficiently specific and reliable to have practical clinical applications in the diagnosis and prognosis of tuberculosis. Nevertheless, the occurrence of high titres in a higher percentage of tuberculous than non-tuberculous patients is too significant to be ignored.

Few studies have been made in children other than those of Anderson and Platou (41) and those included in their series by Fleming, Runyon, and Cummings (9), and others (11, 18). Anderson and Platou, while recognizing limitations in the test, tended to regard it favourably as to its clinical usefulness in children.

Most workers have noted the occurrence of positive reactions in individuals not believed to have had tuberculosis as well as of negative reactions in patients with tuberculosis. The reasons for these false reactions have not been clarified. It is difficult to rule out past subclinical tuberculous infection as a reason for false positives especially in adults. In this respect, further studies in children of different age groups from areas with a low incidence of tuberculosis should be of interest.

Our experience with the hemagglutination test, recorded here, includes studies in infants, children, and adults as well as in a series of experimental animals.

## METHODS

The technique of the hemagglutination test was that described by Middlebrook (24) with few modifications. Sera were inactivated at 65° C. for 3 minutes instead of 56° C. for 30 minutes and were diluted before use with 2 volumes of phosphate-buffered saline instead of 3 volumes. Heterophile antibodies were found to be removed more readily by absorbing each serum three times with unsensitized sheep erythrocytes. The mixture of serum with unsensitized cells was shaken for 20 minutes at room temperature, centrifuged, and the supernatant removed to fresh cells for reabsorption. Serial dilutions of the final supernatant were made in 0.4 ml. volumes to which were added equal volumes of a 0.5 per cent suspension of sensitized sheep cells. Standard concentrated old tuberculin, lot 27-1, prepared by the Connaught Medical Research Laboratories was used to sensitize the erythrocytes instead of the Lederle 4X tuberculin. These two tuberculins were compared by Armstrong and Orlicki

[1] Assisted with funds allocated by the Province of Ontario under the National Health Grants programme of the Department of National Health and Welfare, Ottawa.

(42) and found to be equally sensitive and specific in the performance of this test. Freshly sensitized sheep erythrocytes were prepared for each daily series of tests (43).

The standard controls included tubes to show inagglutinability of the sensitized cells in phosphate-buffered saline, complete absorption of heterophile antibodies using unsensitized cells, and proper cell sensitization using a standard serum with a titre of 1:1024 prepared by immunizing rabbits with B.C.G.

Titres of less than 1:4 were recorded as negative. Those of 1:4 and 1:8 were classified with the negatives as insignificant, and those of 1:16 or over were considered positive.

## RESULTS

### *In children*

Figure 1 shows the hemagglutinin titres found in 165 children.

Sera from 15 newborn infants with erythroblastosis fetalis all gave completely negative reactions.

The other 150 children comprise three groups. The first 50 were under 5 years of age, the second 50 were between the ages of 5 and 14 years. Most of these 100 children were ill with mild upper respiratory infections or various other diseases, but none had evidence of recent or past tuberculosis, and all had negative Mantoux reactions. The third group of 50 children were under 5 years of age, and all had active tuberculosis which was proven by positive cultures and/or guinea pig inoculations in 31. The disease was clinically typical in the remaining 19. The manifestations of tuberculosis were primarily pulmonary in 40, while 10 were cases of tuberculous meningitis with pulmonary lesions.

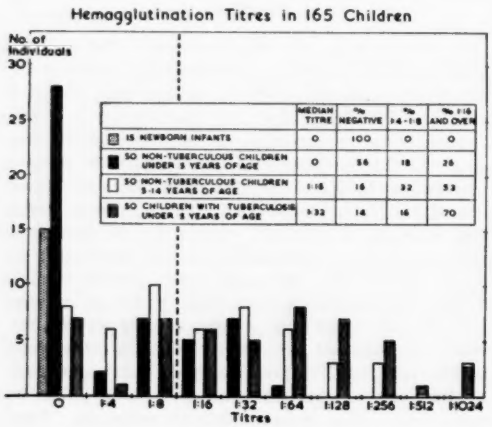


FIGURE 1

In the younger control group the hemagglutinin titre was negative in 28 (56 per cent) and a further 9 showed titres of only 1:4 and 1:8, a 74 per cent incidence of insignificant reactions. There were fewer negatives, 16 per cent, in the older control group, and only 48 per cent altogether with insignificant titres. Both groups, therefore, showed false positive reactions; 26 per cent in the younger and 52 per cent in the older children, a significant difference between the two groups. There were differences as well in the range of titres. The highest found in the younger children was 1:64 (one patient), while in the older group there were 12 at or above this level and up to 1:256.

The reasons for these false positives or for the rising incidence with increasing age are obscure. False positives have been attributed to tuberculin testing. According to Smith and Scott (8), and others, tuberculin testing will stimulate the formation of hemagglutinins, but Colwell and Pitner (23) and Anderson and Platou (41) found that this would not occur readily in non-reactors to tuberculin. Unfortunately all sera could not be collected before Mantoux testing. However, tuberculin testing and serum collection was done similarly in both groups. Moreover, many sera were collected on the same day as the Mantoux test and most within 48 hours of it. Of the 13 false positives in the younger group, 9 were collected before or on the same day as the Mantoux test was done, and all but 2 within 48 hours. Of the 26 false positives in the older group, 13 were taken before or on the same day and another 8 within 48 hours. The six highest titres of 1:128 and 1:256 included two sera collected on the same day, two in 24 hours, one in 48 hours and one in 72 hours after the Mantoux test was done. It is difficult to believe that antibodies in such titres would appear so soon after a single antigenic stimulus. Moreover, antibodies would not be expected so early in a group of tuberculin positive individuals where a "booster dose" effect might be anticipated. In our opinion, Mantoux testing has not affected the results.

In the last group of 50 children, those under five years of age with proven tuberculosis, 70 per cent showed positive titres ranging from 1:16 up to

1 : 1024. This is significantly different from the control group of similar age (26 per cent) and from the older control group (52 per cent) though the latter difference is less marked. The median titre was 1 : 32 in the tuberculous group, but was 1 : 16 in the older non-tuberculous children and less than 1 : 4 in the non-tuberculous groups under five years of age. In the tuberculous group, however, 30 per cent had insignificant titres; 7 or 14 per cent were negative. These false negatives could not be correlated with the extent or type of tuberculosis, nor with the use, early or otherwise, of antibiotic therapy. Of the ten cases with proven tuberculous meningitis which are included in this group, four gave false negative reactions; this finding is in disagreement with the suggestion of Nommik and Meakins (43) that high titres are seen in an unusually high percentage of cases with tuberculous meningitis.

*In adults*

Figure 2 shows the hemagglutinin titres in 200 adults comprising four groups.

There are 50 healthy young adults, blood donors from the hospital transfusion service, without clinical evidence of tuberculosis. A tuberculin test was not done. Insignificant titres were found in 62 per cent of these of which six, or 12 per cent, were negative. However, 38 per cent showed titres ranging from 1 : 16 to 1 : 256. The median for the entire group was 1 : 8. The pattern as regards false positives was very similar to that in the control group of older children, and the reason for it is equally obscure. It is more difficult in these adults to disregard the possibility of previous exposure to the tubercle bacillus.

The second group comprises 50 adults with presumably early tuberculosis. These sera were collected at or shortly after the time when tuberculous disease was first diagnosed. The tuberculosis was not of the chronic fibro-caseous type, and the histories were largely of short duration. There was a sharp contrast with all normal groups. All showed hemagglutinins, with 96 per cent in the significant range of 1 : 16 to 1 : 1024. There were only two (4 per cent) with insignificant titres. This group differs significantly as well from the cases of tuberculosis in young children.

Almost exactly similar to this group is the third comprising 50 adults with chronic fibro-caseous pulmonary tuberculosis. Hemagglutinins ranging in titre from 1 : 16 to 1 : 1024 were found in 94 per cent. Again none were negative, but three or 6 per cent gave insignificant titres of 1 : 8.

Finally there are 50 adults, treated cases of tuberculosis, whose tuberculosis had been considered arrested or "cured" for from one to sixteen years. These sera were collected at a clinic where such patients are examined periodically for evidence of reactivation. Only one serum was completely negative, and 22 per cent showed insignificant results. The range of titres in the 78 per cent with significant hemagglutinins was somewhat lower than in the active cases, ranging up to 1 : 512 only, with a median for the group of 1 : 32.

Hemagglutination Titres in 200 Adults

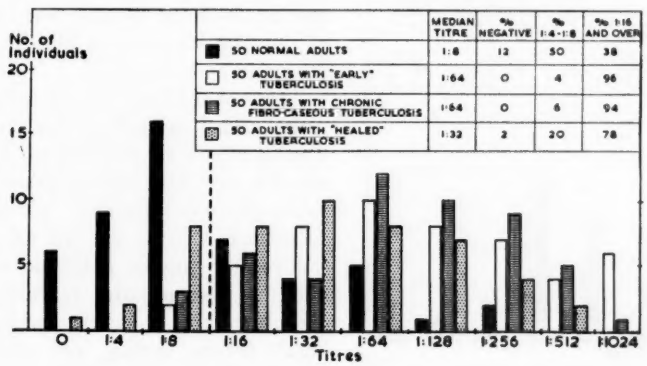


FIGURE 2

The median titre for the previous two groups with active tuberculosis was 1:64. These findings differ significantly both from those seen in all non-tuberculous controls and in adults with active tuberculosis, but are somewhat similar to the results in young children with tuberculosis. The small percentage of arrested cases of tuberculosis with insignificant hemagglutinin titres makes it doubtful that the test is of value as a guide to the activity of the tuberculous process.

*In animals*

Figure 3 shows the hemagglutinin titres in 50 unselected guinea pigs with proven tuberculosis induced by the subcutaneous injection of exudates from tuberculous patients. The sera were collected immediately preceding autopsy of the animals at two months. These titres are compared with those of 50 unselected uninoculated guinea pigs. Hemagglutinins were present in 22 of the controls, and 5 had titres of 1:16. Two of the tuberculous animals failed to show agglutinins, 10 had titres below 1:16, and 38 had titres varying from 1:16 to 1:1024. There was no correlation between titre and extent of tuberculosis, though one of the negative pigs had extensive disease. The difference between the two groups is highly significant. Nevertheless it would seem that in dealing with guinea pigs one might expect false positive reactions in 10 per cent of uninfected animals and false negative reactions in 24 per cent of tuberculous ones.

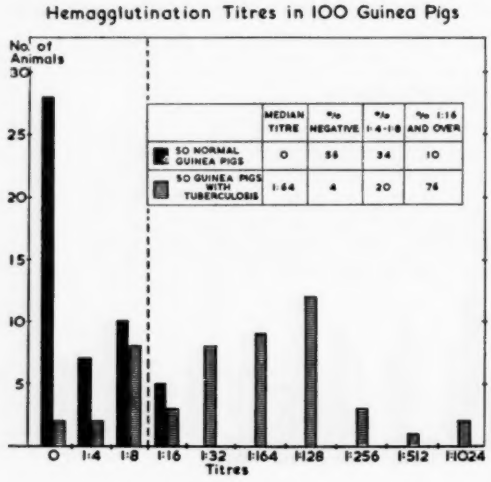


FIGURE 3

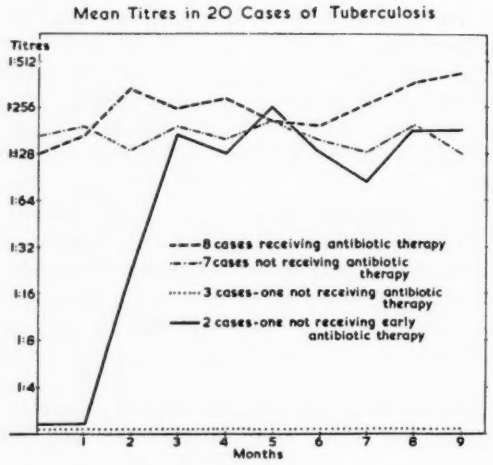


FIGURE 4

*Miscellaneous observations in children and animals*

Twenty selected children with active tuberculosis (pulmonary and meningeal) were followed by monthly hemagglutinin titrations for nine months. Figure 4 shows the means of the titres set out in four groups.

Five children gave negative hemagglutination reactions when first tested. These false negative reactions may have been found because the initial samples of sera were taken too early in the course of the disease.

Two of the five subsequently developed hemagglutinins to titres of 1:128; the positive response occurred in one after two months even though specific antibi tic therapy was begun at the time of the first sampling of blood. The clinical response in both was good.

Three of the five children failed to develop hemagglutinins throughout the nine-month period. Two of these three received specific chemotherapy, and this may have been responsible for their failure to give a positive test. The third child, whose tuberculous disease was not clinically severe, did not receive specific antibiotic or chemotherapy, and the false negative reactions persisted even though cultures of gastric washings grew tubercle bacilli before, during, and after the period of observation.

Fifteen of the children had hemagglutinins initially. Eight of the 15 received specific antibiotic and chemotherapy, seven did not. The titres of most individuals remained at a fairly constant level during the nine-month period. The mean titres of the two groups (shown in Figure 4) were maintained at a fairly constant and similar level throughout. There were no detectable differences between those receiving and those not receiving antibiotic and chemotherapy and no correlation with clinical response to therapy.

The Coombs antiglobulin technique was used in 45 selected children following the suggestion of Meynell (40). This technique was investigated to find out if positive reactions in tuberculous cases could be differentiated from false positive reactions in non-tuberculous patients by demonstrating significantly higher titres of "incomplete" antibodies in the sera of patients with tuberculosis. It was also used to determine if "incomplete" antibodies could be detected in the sera of patients with tuberculosis giving false negative hemagglutination reactions.

The red blood cells in the tubes failing to show agglutination were washed thoroughly with buffered saline and mixed with a rabbit anti-human-globulin serum found satisfactory for Rh work. This serum failed to agglutinate control unsensitized or tuberculin sensitized sheep cells.

The procedure increased the titres of some sera, but not all. Increases could be demonstrated in only 28 of the 45 cases; the increase was usually 2-fold to 8-fold and rarely 128-fold. Such increases tended to occur more frequently in sera from tuberculous cases than from controls, but this was inconstant. Of particular interest were the five tuberculous cases referred to earlier with negative initial hemagglutinin tests. Only three of the five were positive by the Coombs method; the titre in one was 1 : 16 and in two it was 1 : 128. All five cases were investigated serologically for nine months. One child receiving chemotherapy remained negative throughout to both tests. Two children remained negative throughout by the usual hemagglutinin test but showed titres of 1 : 128 for nine months by the Coombs technique. The remaining two patients developed hemagglutinins subsequent to the first examination. In one of these two the Coombs test always showed titres from two to eight times the titre of the hemagglutination test, but in the second child the Coombs test never showed a titre higher than the hemagglutinin titre.

Tuberculin testing was discussed earlier as a possible reason for false positive reactions in non-tuberculous children. Tests were done in guinea pigs to determine how readily a hemagglutinin response might occur following the injection of tuberculin. Nine guinea pigs with negative hemagglutination tests were given repeated subcutaneous injections of 50.0 mg. of old tuberculin at two-week intervals for 16 weeks. This dose is 1000 times the quantity used for routine Mantoux testing in our Hospital. At 2 weeks, four animals were negative, three had titres of 1 : 4, and two had titres of 1 : 16. At 4 weeks, four of the nine guinea pigs remained negative, three had titres below 1 : 16, and the titres of two had increased to 1 : 64. Not until the end of 8 weeks (four injections) were positive tests obtained in all animals; the highest titre was 1 : 64, and five had titres of only 1 : 4 or 1 : 8. The titres were much the same at 14 and 16 weeks. The hemagglutinin response to tuberculin injections in guinea pigs thus is slow and poor.

An attempt was made to determine if antigens other than those derived directly from the tubercle bacillus could stimulate the formation of antibodies which would agglutinate tuberculin sensitized erythrocytes. Groups of young guinea pigs with initially negative hemagglutination tests were given three subcutaneous injections at two-week intervals of various antigens, and blood samples were collected two weeks after the last injection. Thirteen pigs were given a mixed formalin killed vaccine made from freshly isolated nasopharyngeal strains of *Staphylococcus pyogenes, Diplococcus pneumoniae, Streptococcus pyogenes* (Lancefield Group A.), *Hemophilus influenzae* (Type b.), *Streptococcus* sp. (viridans), *Neisseria* sp., and diphtheroids. All remained negative to the hemagglutination test but one which had a titre of 1 : 8. Eight guinea pigs received injections of fluid diphtheria toxoid. Four developed titres of 1 : 32, two had titres of 1 : 16, and only two remained negative to the test. Seven guinea pigs were given a vaccine prepared from a saprophytic orange-pigmented mycobacterium which has been grown on several occasions from gastric washings from infants and children. All became

positive to the hemagglutination test with the titres ranging from 1 : 32 to 1 : 256. Two were at a titre of 1 : 256, three at 1 : 128, one at 1 : 64, and one at 1 : 32.

## DISCUSSION

There are obvious and significant differences in the incidences of positive hemagglutination tests at a titre of 1 : 16 or more between the tuberculous and non-tuberculous cases in any age group. These differences are also apparent in the ranges of titres.

While this is true, the test has little or no practical diagnostic value, except perhaps in those few cases of tuberculosis with exceptionally high titres, because of the occurrence of so many false negative reactions in the tuberculous group and false positive reactions in the non-tuberculous group. False reactions in both directions were obtained as well in control and tuberculous guinea pigs. The arbitrary dividing point chosen between non-significant and significant titres, levels of 1 : 8 to 1 : 16, seemed to fit best the group as a whole, though a 1 : 4 to 1 : 8 division would have produced better differentiation in adults as regards insignificant reactions in active cases. The addition of a Coombs technique did not make the test more specific, though it is of interest that a few, but not all, tuberculous cases with negative hemagglutination tests gave moderately high titres by this added procedure.

The hemagglutination test lacks practical prognostic significance as an index of the activity of the tuberculous process, even though there was a somewhat significant difference in the incidence of negative or insignificant titres between those adults with early or chronic active tuberculosis and those considered clinically as "cures." The pattern of the titres in "healed" adult tuberculosis differed little from that in active tuberculosis in children under five years of age. Children with active tuberculosis who received clinically effective specific antibiotic and chemotherapy for nine months failed to show a tendency to have lower titres. This period of observation may have been too short.

The reasons for false negative results in proven cases of tuberculosis are obscure. False negatives were of significantly greater frequency in early childhood tuberculosis than in adult cases. From our series, this could not be attributed to early specific antibacterial therapy or to massive infection. It would appear that antibody response is poor in certain individuals, and this occurs more frequently in early childhood.

Of great interest are the false positive reactions obtained in non-tuberculous humans as well as in non-tuberculous animals, and of particular interest are the frequencies of such reactions in different age groups. The incidence of false positive tests rose from 0 per cent in newborn infants to 26 per cent in children under five years of age and to 52 per cent in children of five years or over while the incidence of completely negative tests fell in the three groups from 100 per cent to 56 per cent to 16 per cent, respectively. The adult controls differed little from the group of older children. These positives cannot be explained by tuberculin testing in the control children. It is difficult to rule out past sub-clinical tuberculous infection in adults. This factor, in our opinion, may be discounted in the young children. The probability of exposure at their age and in a community with a relatively low incidence of tuberculosis is minimal. Furthermore, clinical evidence of tuberculosis was absent, and Mantoux tests were negative.

Whether the increasing incidence of false positive tests from infancy to age 14 is an expression of some "maturation factor" of a completely non-specific nature is not known. Our findings, including the better antibody response in tuberculous adults, suggest a "secondary" or "booster" effect of an antigen to which individuals have been exposed on one or more occasions. The test appears to have a high degree of specificity in cases of tuberculosis. But in the non-tuberculous controls especially in children, the findings suggest an immunological stimulation by an antigen or antigens having some common factor with the tubercle bacillus. In this respect, the demonstration that antibodies capable of agglutinating tuberculin sensitized erythrocytes could be produced in guinea pigs by antigenic substances not prepared from the tubercle bacillus, such as fluid diphtheria toxoid and a suspension of a saphrophytic mycobacterium, is of interest. Antigenic exposure to diphtheria toxoid is common in childhood. Exposure under natural conditions to this particular mycobacterium to a degree which would result in the formation of hemagglutinins cannot be disregarded as a possible explanation of false positive hemagglutination tests. This could occur during the course of upper respiratory infections caused

by other micro-organisms, or for other reasons, and the chance of such antigenic exposure might increase with age. Factors such as these could account for the increasing incidence with age of false positive hemagglutination tests and the better antibody response seen in adults with tuberculosis.

If this thesis proves valid, namely, that the defects of the test are due to antigens shared by the tubercle bacillus (or present in tuberculin) and common substances to which individuals may be exposed, there is little hope for clinical usefulness of the test until antigens specific for pathogenic strains of M. tuberculosis are found which are capable of sensitizing erythrocytes.

## SUMMARY

1. The Middlebrook-Dubos hemagglutination test, using tuberculin sensitized erythrocytes, has been studied in children, in adults, and in a series of experimental animals.

2. A hemagglutinin titre of 1 : 16 or more was found in only 70 per cent of children under five years of age with active tuberculosis and in 95 per cent of adults with active tuberculosis. The incidence of false negative reactions decreased with age.

3. A hemagglutinin titre of 1 : 16 or more was found in 0 per cent of newborn infants, in 26 per cent of non-tuberculous children under five years of age, in 52 per cent of non-tuberculous children 5 to 14 years of age, and in 38 per cent of normal adults. The incidence of false positive reactions increased with age.

4. False positive and false negative reactions were also found to occur in experimental animals.

5. The test had very limited value in the laboratory diagnosis of tuberculosis because of the frequency of false positive reactions in non-tuberculous individuals and of false negative reactions in active cases of tuberculosis especially in children.

6. A Coombs modification of the hemagglutination test did not make the results more specific.

7. The hemagglutination test failed to differentiate accurately between active cases of tuberculosis and "healed" cases or to detect clinical regression of the disease during nine months of specific antibiotic and chemotherapy.

8. An antibody capable of agglutinating tuberculin sensitized sheep erythrocytes was found to develop in all of seven guinea pigs injected with a saprophytic mycobacterium, in six of eight guinea pigs injected with diphtheria toxoid, and in one of thirteen guinea pigs injected with a vaccine of mixed nasopharyngeal bacteria.

## ACKNOWLEDGMENTS

The authors are grateful to Professor P. H. Greey and to Dr. A. R. Armstrong, Director of Laboratories, The Mountain Sanatorium, Hamilton for most helpful advice; to Dr. H. T. McClintock, Director of the Chest Clinic, The Gage Institute, Toronto, for providing the sera from adults with arrested tuberculosis and to Dr. A. G. S. Heathcote, Director of Laboratories, The Toronto Hospital for Tuberculosis for sera from adults with active tuberculosis.

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# SURFACE AS A QUANTITATIVE FACTOR IN PROTHROMBIN UTILIZATION [1]

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(Submitted for publication May 20, 1954; accepted June 30, 1954)

The accelerating effect of foreign surfaces on coagulation of whole blood has been recognized for many years. In 1863, Lister (1) was ". . . aware of the great influence exerted upon the blood by exposure for a very short time to a foreign solid . . . ," and concluded, "The real cause of the coagulation of the blood, when shed from the body, is the influence exerted upon it by ordinary matter. . . ." Since the original observations of Lister, many subsequent investigators have studied this phenomenon (2–15).

In the experiments to be described, the rate of prothrombin utilization was employed as a measure of the effect of foreign surface on the blood coagulation mechanism. Blood and plasma from normal human subjects and from patients with hemophilia and thrombocytopenia were exposed to differing glass surface areas, and prothrombin utilization was measured.

## METHODS

*Methods of obtaining and handling blood:* Approximately 60 ml. of blood were drawn from the antecubital vein of human subjects using sterile, non-silicone treated 18 gauge needles and silicone [4] treated syringes lubricated with silicone oil.[5] In order to expose this blood to various areas of foreign surface, 2.0 ml. portions of the blood were measured in duplicate directly from the syringe into 13 × 100 mm. test tubes which had been prepared in different ways. Some tubes were silicone-treated, others were plain glass (*i.e.*, chemically clean, dry, "Pyrex" glass), and still others were plain glass tubes containing weighed amounts of crushed glass.[6] The silicone treated tubes were left undisturbed. Some of the plain glass tubes were left undisturbed, others were completely inverted 25 times with a parafilm [7] stopper by the use of a gentle rocking motion, and still others were vigorously shaken for two minutes with a 00 rubber stopper. The tubes containing crushed glass were vigorously shaken for two minutes with a 00 rubber stopper. All of the tubes were then incubated in a water bath maintained at 37° C. for measured periods of time. The time of incubation was considered to have begun when the blood was first seen entering the syringe. At the end of the desired period of incubation, coagulation was terminated by the addition of 0.2 ml. of M/10 sodium oxalate. The clot, if any, was completely broken and the tubes shaken. The supernatant plasma or serum was obtained by centrifugation at 2,500 rpm. for 5 to 10 minutes. The residual prothrombin was determined by the one-stage procedure, and the remaining plasma or serum was stored at − 20° C. until analyzed by the two-stage method. To serve as a control, 4.5 ml. of the original blood specimen were mixed with 0.5 ml. of M/10 sodium oxalate immediately after withdrawal, and the plasma was obtained by centrifugation at 2,500 rpm. for 5 to 10 minutes. The one-stage prothrombin time was determined, and the remaining plasma was stored at − 20° C. until analyzed by the two-stage procedure.

*Preparation of plasma with varying platelet levels:* Using silicone-treated syringes lubricated with silicone oil, blood was obtained from normal human subjects without the use of an anticoagulant. Plasma with varying platelet levels was prepared by differential centrifugation at low temperatures as previously described (16).

*Two-stage prothrombin determinations* were performed by a modification of the procedure of Ware and Seegers (17). This procedure was modified as follows: 1) M/10 sodium oxalate was used as an anticoagulant instead of 3.2 per cent sodium citrate; 2) the procedure was carried out at 37° C.; and 3) the fibrinogen used was prepared from human plasma by the cold precipitation method of Ware, Guest, and Seegers (18). This fibrinogen was further purified by modifying the method of

[1] This work was carried out under Contract AT (30-1) 1208 between The Atomic Energy Commission and The Johns Hopkins University, and was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health.

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[4] General Electric Company, Dri-film, SC-87 (formerly 9987).

[5] General Electric Company, Silicone Oil, SF-96 (200) (formerly 9996-200).

[6] Crushed glass was prepared from chemically clean, dry, "Pyrex" glass ground in a mortar and pestle. The crushed glass used had a particle size that passed a No. 50 mesh screen and that was retained by a No. 100 mesh screen.

[7] Parafilm grade "M", Marathon Corporation.

Laki (19). The additional purification consisted of washing the product with oxalated saline four to five times in the cold, dissolving at room temperature in oxalated saline, precipitating with 25 per cent saturated ammonium sulfate, re-dissolving in buffer at pH 7.3, dialyzing against barbital buffer at 5° C., filtering with pressure through a Seitz filter using 20 per cent asbestos pads, and storing at − 20° C. At the beginning of these experiments, 250 ml. of citrated plasma were obtained from a normal subject and stored in 2.0 ml. portions at − 20° C. This plasma served as a reference standard for the two-stage procedure, and daily checks for accuracy of this procedure were performed on this plasma. In these experiments appropriate correction was made for dilution of the plasma or serum with anticoagulant as determined by the hematocrit values.

*Residual prothrombin by the two-stage procedure* was determined without modification of the above procedure. It should be noted that when the residual prothrombin was less than 10 per cent of the control, accurate measurement of prothrombin could not be made by this technique.

*One-stage prothrombin times* were determined in duplicate at 37° C. by a modification of the procedure of Quick (20), using 0.1 ml. of the control oxalated plasma, 0.1 ml. of a standardized rabbit brain thromboplastin, and 0.1 ml. of M/10 calcium chloride. The normal range is 16 to 18 seconds.

*Residual prothrombin by the one-stage procedure* was determined in duplicate at 37° C. by a modification of the procedure of Quick and Favre-Gilly (21). The clotting time of a mixture of 0.1 ml. of the test oxalated plasma or serum, 0.1 ml. of barium sulfate adsorbed plasma from a normal subject, 0.1 ml. of a standardized rabbit brain thromboplastin, and 0.1 ml. of M/25 calcium chloride was determined. Prothrombin time of unclotted plasma using this technique did not differ significantly from the results of the one-stage prothrombin test described above.

*The volume of packed cells,* expressed in per cent, was determined in Wintrobe hematocrit tubes.

*Platelet counts* were performed by the phase contrast technique of Brecher and Cronkite (22) in which 1 per cent ammonium oxalate is used as the diluent. A dilution of 1:100 was used when the platelet level was approximately normal, whereas thrombocytopenic specimens were diluted 1:20. Platelet counts on specimens of platelet-poor plasma were performed by observing the undiluted plasma in the counting chamber using the phase contrast microscope.

*Whole blood clotting times* were determined by a modification of the method of Lee and White as previously described (23).

*Tests for the detection of circulating anticoagulants* were performed by a previously described method (24).

## RESULTS

### *Effects of varying glass surface area on the rate of prothrombin utilization in whole blood*

*Normal blood:* In 11 experiments on normal human subjects, blood was obtained by a single venipuncture and distributed into a series of duplicate tubes prepared to provide various areas of glass surface. These tubes were incubated at 37°

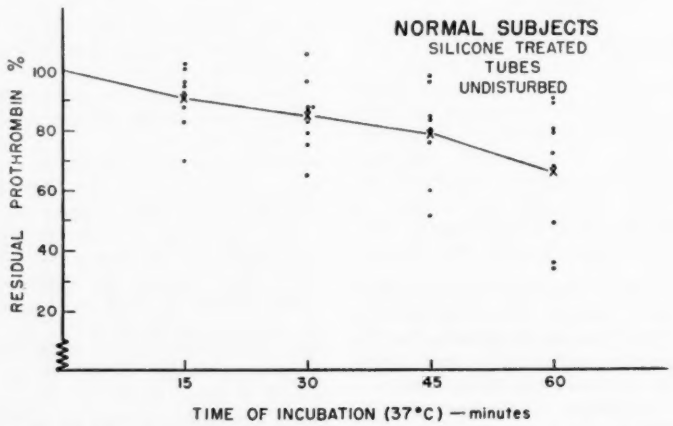


FIG. 1. RESIDUAL PROTHROMBIN AS DETERMINED BY THE TWO-STAGE PROCEDURE EXPRESSED IN PER CENT OF THE PRE-INCUBATION VALUE FOR EACH SUBJECT

Each point in the graph represents the mean of duplicate determinations. The crosses connected by the solid line represent the mean of all the determinations at each time interval.

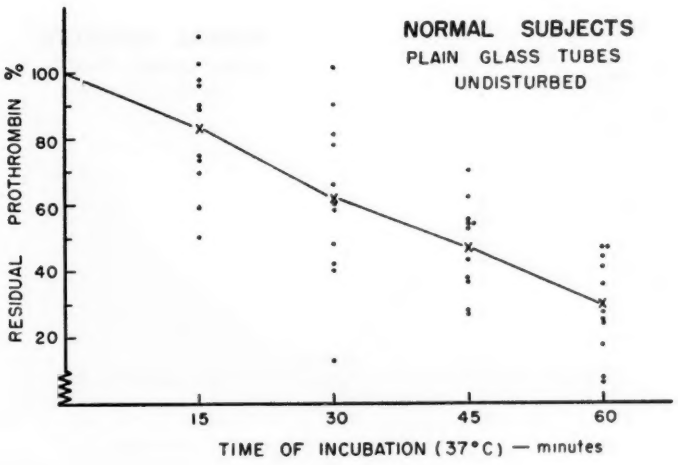


FIGURE 2

The points on this graph represent the mean of duplicate determinations on blood specimens from the same venipuncture as those shown on Figures 1 and 3. Only the surface to which the blood was exposed was changed. The residual prothrombin was measured as in Figure 1.

C. for periods up to 60 minutes. Coagulation was terminated at the end of the desired interval by the addition of oxalate, and the residual prothrombin was determined by the two-stage procedure.

When normal blood was incubated in undisturbed silicone-treated tubes, the rate of prothrombin utilization tended to be slow (Figure 1). However, there was considerable variation in the results of the individual determinations, and after 60 minutes of incubation, the residual prothrombin varied between 34 and 90 per cent. It is apparent that under these conditions, normal blood may fail to show significant prothrombin utilization.

When normal blood was incubated in undisturbed plain glass tubes, the rate of prothrombin utilization was increased (Figure 2). Again, there was considerable variation in the results of the individual determinations, and after 60 minutes of incubation, the residual prothrombin varied from less than 10 to 47 per cent.

Gentle inversion of the plain glass tubes at the beginning of the period of incubation greatly accelerated the rate of prothrombin utilization (Figure 3). Under these conditions, utilization of prothrombin was virtually complete in every instance in 45 minutes of incubation. In many other experiments, agitation of normal blood in plain glass tubes or in tubes containing crushed glass invariably led to complete utilization of prothrombin after 60 minutes of incubation.

Other experiments were designed to determine whether transient exposure of blood to glass surface would lead to continuing utilization of prothrombin. Two ml. portions of whole blood from a normal individual were measured into a series of plain glass and silicone-treated tubes. Some of the glass tubes were inverted 25 times with a parafilm stopper, and others were shaken for two minutes with a rubber stopper. Immediately following this agitation, and within three minutes after initial exposure of the blood to glass, duplicate samples were transferred to silicone treated tubes. Other duplicate samples were left in the original glass and silicone-treated tubes. All tubes were then incubated, undisturbed for 60 minutes at 37° C. Coagulation was terminated at various times by the addition of oxalate, and residual prothrombin was determined by the two-stage procedure. Blood transiently exposed to glass showed little or no change in the level of prothrombin immediately after exposure to glass, but during subsequent incubation in silicone-treated tubes, prothrombin utilization was as rapid as in blood stored in contact with glass throughout the pe-

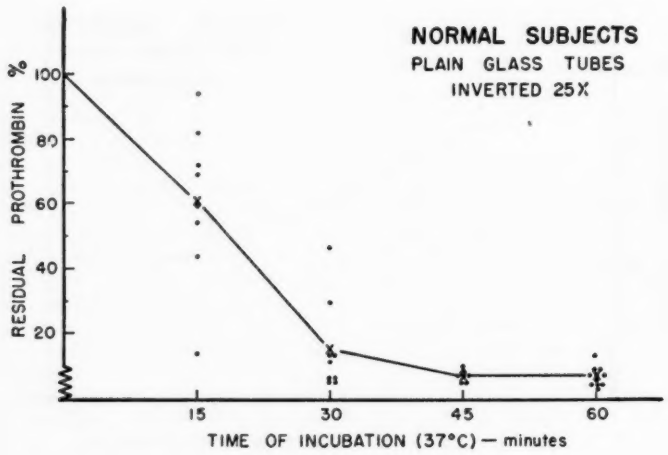


FIGURE 3

Blood specimens were obtained from the same venipunctures as in the studies shown in Figures 1 and 2. The glass tubes were gently inverted 25 times after the introduction of the blood, and then were allowed to stand undisturbed during the remainder of the incubation period. The undulating portion of the ordinate indicates that values of less than 10 per cent generally could not be accurately measured by the two-stage procedure.

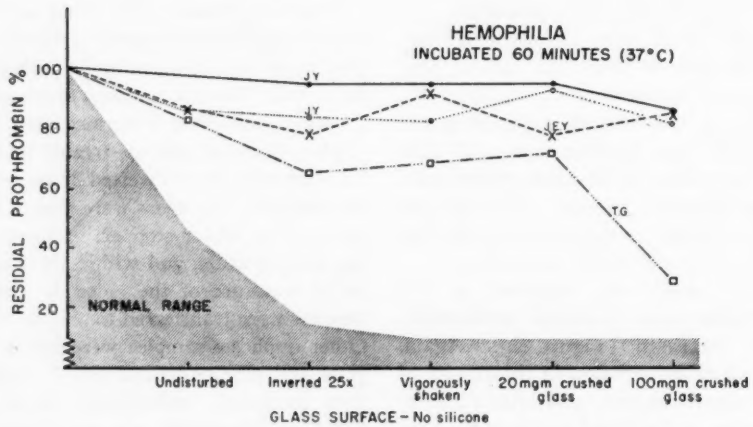


FIG. 4. THE RESULTS OF FOUR EXPERIMENTS ON THE BLOOD OF THREE HEMOPHILIC PATIENTS

Each point on the graph represents the mean of duplicate determinations of residual prothrombin as measured by the two-stage procedure. The time of incubation was held constant at 60 minutes. The degree of exposure of the blood to glass surface area was varied as indicated on the abscissa. In this and in Figures 5–8 the units on the abscissa have no numerical significance, since surface exposure was not measured in absolute terms.

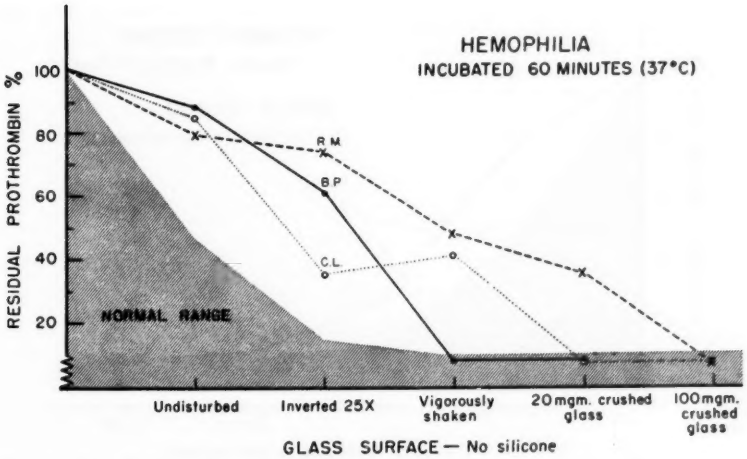


FIG. 5. THE RESULTS OBTAINED ON THE BLOOD OF THREE ADDITIONAL HEMOPHILIC PATIENTS

The procedures employed were identical to those used in obtaining the data for Figure 4.

riod of incubation. The blood that was exposed only to the silicone-treated tubes, without any exposure to glass, utilized much less prothrombin.

It is apparent from these data that the rate of prothrombin utilization in normal blood is directly related to glass surface area to which the blood is exposed. It is interesting to note that a mean residual prothrombin value of approximately 70 per cent was found in normal blood incubated in the silicone-treated tubes for 60 minutes, in the undisturbed plain glass tubes for 30 minutes, and in the inverted plain glass tubes for 15 minutes.

*Hemophilic blood:* Blood was obtained from six hemophilic patients and distributed into a series of duplicate glass tubes which were incubated at 37° C. for 60 minutes. Some of the tubes were left undisturbed throughout the period of incubation. Others were inverted 25 times or were vigorously shaken for two minutes and then left undisturbed. Some of the tubes that were vigorously shaken contained weighed amounts of crushed glass. After 60 minutes of incubation, coagulation was terminated by the addition of oxalate and the residual prothrombin determined by the two-stage procedure.

The results of four experiments on the blood of three hemophilic patients are shown in Figure 4. In none of these studies did the prothrombin utilization reach the normal range even when the blood was exposed to large glass surface areas. Nevertheless, appreciable prothrombin utilization did occur in one instance.

In identical studies on the blood of three other hemophilic patients, prothrombin utilization was directly related to the glass surface area, and when glass surface area was sufficiently large, utilization of prothrombin was complete (Figure 5).

All six of these hemophilic patients had classical manifestations of the disease. Clinically all but one (B. P.) had severe hemophilia. None of these patients had recently been transfused or received antihemophilic preparations. In each case, tests for the detection of circulating anticoagulants gave negative results. The blood of patient J. Y. (Figure 4) failed to correct the prothrombin utilization defect of all of the other five patients. The coagulation defect in each case was corrected *in vitro* by a source of antihemophilic globulin believed to be deficient in PTC (Christmas) factor.

*Thrombocytopenic blood:* Blood was obtained from 10 patients with platelet counts ranging between 2,000 and 23,000 per cmm. The blood was handled in the same manner as that described for hemophilic blood.

The results of this study are shown in Figure

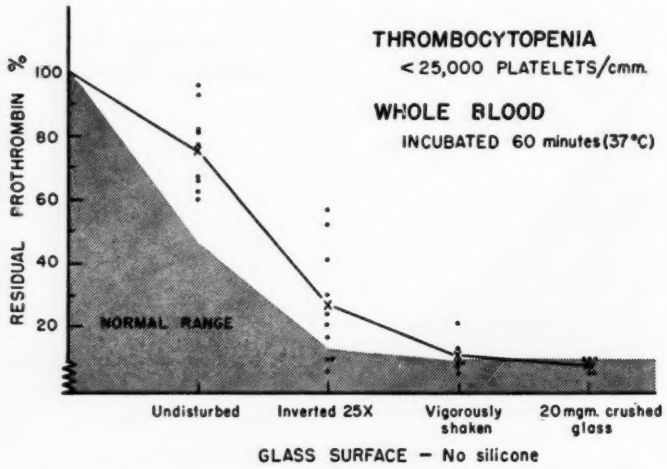


FIG. 6. THE RESULTS OBTAINED ON THE BLOOD OF 10 PATIENTS WITH THROMBOCYTOPENIA

Each point on the graph represents the mean of duplicate determinations of residual prothrombin as measured by the two-stage procedure. The time of incubation was 60 minutes. The degree of exposure of the blood to glass surface area was varied as indicated on the abscissa. The crosses connected by the solid line represent the mean of all of the determinations at each glass surface exposure.

6. It is apparent that only when the blood was left undisturbed in plain glass tubes were the results uniformly outside the normal range. Following simple inversion of the glass tubes 25 times, prothrombin utilization in three instances became as rapid as the normal. When exposure to glass surface was further increased, prothrombin utilization was complete in every instance.

Some of these thrombocytopenic patients were found to have prolonged whole blood clotting times. In these cases tests for the detection of circulating anticoagulants were negative.

*Effect of varying glass surface area and platelet concentration on the rate of prothrombin utilization in plasma*

Plasma with varying platelet levels was prepared from the blood of fasting normal subjects without the use of an anticoagulant. In each experiment, blood obtained by a single venipuncture from one subject was employed. Two ml. portions of the plasma were distributed in test tubes providing varying exposure to glass surface. After 60 minutes incubation at 37° C., coagulation was terminated by the addition of 0.3 ml. of M/10 sodium oxalate and the residual prothrombin was measured. In order to determine whether prothrombin utilization had occurred as a result of the manipulation involved in the preparation of the plasma, two types of controls were employed. The first control was plasma obtained from blood which was oxalated immediately following withdrawal from the vein. The second control was plasma which was oxalated after centrifugation had been performed but before the beginning of the period of incubation. In every experiment there was no significant difference between the prothrombin concentration of the two control specimens. The time of incubation was considered to have begun when the test plasma was introduced into the tubes at 37° C. Therefore, the period of incubation was considered to have begun in these experiments after the blood had been centrifuged.

A representative experiment is shown in Figure 7. It is apparent that when the glass surface area was constant, the rate of prothrombin utilization was directly related to the platelet concentration.

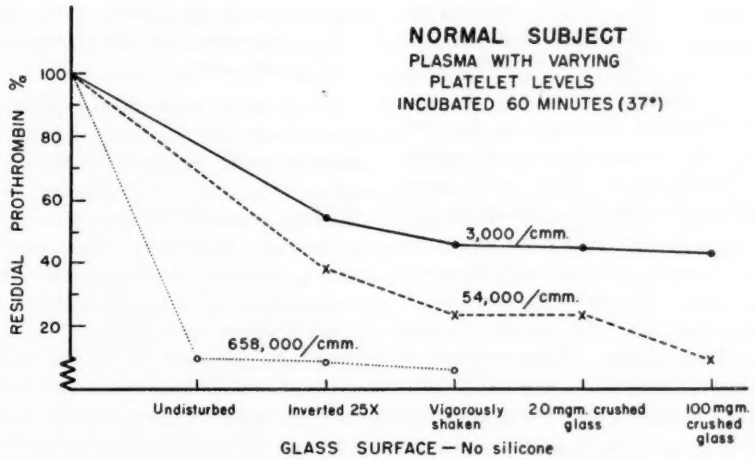


FIG. 7. PLASMA SAMPLES WITH VARYING PLATELET LEVELS PREPARED FROM BLOOD OBTAINED BY A SINGLE VENIPUNCTURE FROM A NORMAL FASTING SUBJECT

Residual prothrombin expressed in per cent of the control is plotted along the ordinate. The degree of exposure to glass surface area is plotted along the abscissa. All samples were incubated for 60 minutes at 37° C.

Conversely, when platelet concentration was constant, the rate of prothrombin utilization was directly related to glass surface area.

In other experiments, prothrombin utilization of plasma that was virtually platelet-free was studied. Platelet-poor plasma obtained as in the preceding experiments was re-centrifuged at 17,500 to 22,000 G for 10 minutes at 4°C. Plasma prepared in this way contained less than 100 platelets per cmm. as determined by direct observation of

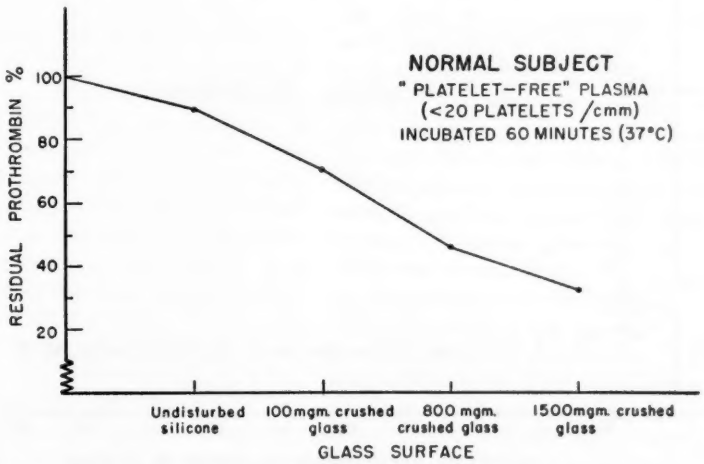


FIG. 8. "PLATELET-FREE" PLASMA PREPARED FROM BLOOD OF A NORMAL FASTING SUBJECT

Residual prothrombin expressed in per cent of the control is plotted along the ordinate. The degree of exposure to glass surface area is plotted along the abscissa. All samples were incubated for 60 minutes at 37° C.

the undiluted plasma in the counting chamber using the phase contrast microscope. Two ml. portions of plasma were exposed to varying glass surface areas as described above, and the rate of prothrombin utilization measured by the two-stage procedure. The results of a typical experiment are shown in Figure 8. When such "platelet-free" plasma was incubated in silicone-treated tubes for periods up to two hours, no significant utilization of prothrombin occurred, and the specimens remained fluid. This indicates that the coagulation process had not been initiated during the preparation of the plasma. When portions of the same plasma were placed in contact with glass surface, solid clots were formed, and prothrombin utilization was directly related to the degree of exposure to glass surface. When the glass surface area was very large, coagulation occurred within two minutes, and approximately two-thirds of the prothrombin was utilized within 60 minutes. In no experiment involving "platelet-free" plasma was complete prothrombin utilization observed. This was true even when the "platelet-free" plasma was vigorously shaken with 3,000 mgm. of crushed glass and incubated for periods up to two hours.

*Comparison of the results of one-stage and two-stage procedures for the measurement of residual prothrombin*

In all of the experiments that have been described, the data pertaining to the residual prothrombin were obtained by the two-stage procedure. In every experiment, one-stage prothrombin consumption tests also were performed on the same specimens. One-stage tests always were performed 30 minutes after the conclusion of the period of incubation and prior to freezing of the samples for storage. The relationship of the results obtained by the one-stage procedure and by the two-stage procedure is shown in Figure 9. It is apparent that regardless of the source of the specimen or the manner in which the specimen was handled, prothrombin utilization was detectible by the one-stage method only when the two-stage test indicated that less than 15 per cent of the prothrombin remained.

## DISCUSSION

The degree of exposure of normal blood and plasma to a glass surface has been demonstrated to have a quantitative influence on the rate of pro-

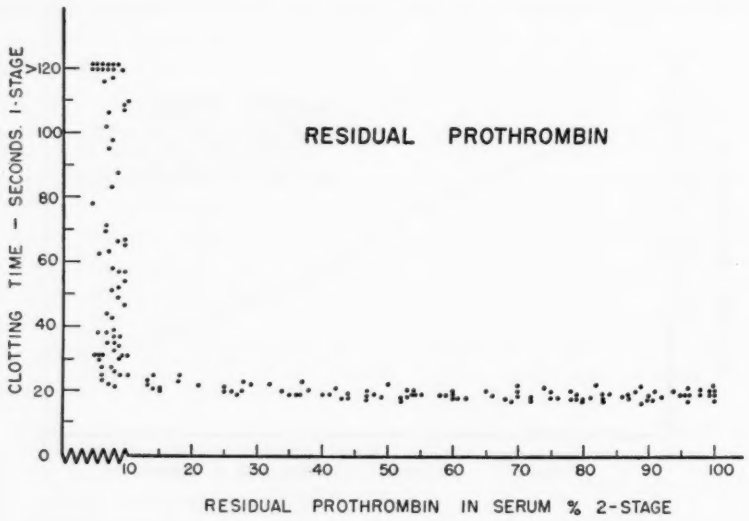


FIGURE 9

In all of the experiments described residual prothrombin was estimated both by the one-stage and two-stage procedures. This graph shows the relationship between the results obtained by the two methods.

thrombin utilization. Brief exposure of blood to glass surface initiates a process which leads to continuing prothrombin utilization even though little prothrombin has been utilized during the actual period of contact with glass. These studies confirm, in a precise manner, the observations of Lister (1).

Lister stated that contact of blood with a foreign surface induced ". . . a mutual reaction between its solid and fluid constituents, in which the corpuscles impart to the liquor sanguinis a disposition to coagulate." Most subsequent investigators, including many current workers, have agreed that the coagulant effect of foreign surface is mediated by the formed elements, particularly the platelets. An impressive array of recent evidence has shown that platelets are quantitatively concerned with the rate of prothrombin utilization in shed blood (13, 25–27). This relationship is confirmed by the present studies which, however, point out that at a given platelet level the rate of prothrombin utilization is directly related to the degree of exposure to a glass surface. The concept of a "critical platelet level" which is optimal for prothrombin utilization is not supported by the current work, since complete prothrombin utilization will occur in whole blood at any platelet level, when the blood is exposed to an appropriate foreign surface.

Unrefuted evidence has been presented by several investigators (3, 5–9, 13, 14) showing that cell-free plasma does contain all of the necessary constituents for coagulation, and that it will clot on contact with foreign surfaces. The current experiments again demonstrate that cell- and "platelet-free" plasma does clot, and furthermore, demonstrate that the rate of prothrombin utilization during clotting of such plasma is a function of the degree of exposure to glass surface. The earlier work has been criticized because the test plasma was not shown to be platelet-free since platelet counts were not performed (1, 3, 5–7), or the methods of counting platelets made it impossible to evaluate the actual platelet levels (8). In more recent work (14), and in the current experiments, direct observation of undiluted plasma samples using the phase contrast microscope have eliminated these objections. A further criticism has been that "platelet-free" plasma may clot because the manipulations involved in its preparation initiate prothrombin conversion by damaging the platelets. This is refuted by the demonstration that prothrombin utilization in such plasma does not occur if the plasma is protected from contact with glass surfaces.

Observations using the phase contrast microscope have been reported (14) showing that "platelet-free" plasma will form fibrin at the point of contact with glass particles in the absence of platelets. That this is true clotting and not defibrination by physical forces is shown by prothrombin utilization studies in such a system. That shaking blood or plasma with crushed glass does not *per se* denature or remove prothrombin is shown by the fact that hemophilic blood which has been incubated following vigorous shaking with crushed glass may show no change in the level of prothrombin.

The exact mechanism of action of glass surface in initiating coagulation is not known. Presumably contact with glass results in the development of thromboplastic activity. Whether this effect is due to an alteration of a specific plasma protein (8), to adsorption of an inhibitor (9), or to some other mechanism is not clear. It has been shown that coagulation of a globulin fraction of "platelet-free" plasma may be accelerated by contact with glass, but the component necessary for this effect was not identified (28). In hemophilia the elaboration of thromboplastic activity in blood and plasma after exposure to glass is impaired. This suggests that the antihemophilic globulin itself may be the site of the alteration. However, this has not been proved, and the blood of some patients with severe hemophilia may demonstrate complete prothrombin utilization when exposed to sufficient glass surface area. It seems likely that several plasma constituents are involved in the production of thromboplastic activity, and that a deficiency of any one might impair the rate of development of this activity. The fact that coagulation of blood or "platelet-free" plasma is hastened by dilution with saline or buffer solutions (14, 29) suggests that a clotting inhibitor is present, but it remains to be proved that the effect of glass surface is brought about by the removal of such an inhibitor.

The present observations have an important bearing on the use of "prothrombin consumption tests" for diagnostic purposes. In the perform-

ance of such tests it is clear that accurate control of exposure of blood to foreign surface is required, if results are to be meaningful. Normal blood may fail to utilize an appreciable amount of prothrombin, if exposure to foreign surface is minimal. On the other hand, in the presence of some coagulation defects prothrombin utilization may be complete if exposure to glass surface is large. When blood is permitted to stand in glass tubes, it is clear that even minor alterations in the manner of handling the tubes results in significant changes in the exposure of the blood to glass. The wide variation in prothrombin utilization in blood incubated in "undisturbed" glass tubes is probably accounted for by this fact. It was hoped in the present study that a single set of experimental conditions could be found which would exaggerate the differences between, and decrease the individual variations of normal and abnormal blood. By moderately increasing the glass surface exposure, hemophilic blood is more readily distinguished from normal blood, but such increased exposure may mask the defect in thrombocytopenic blood. In the present experiments prothrombin utilization could be detected by the one-stage method only when the two-stage procedure indicated that less than 15 per cent of the original prothrombin remained.

## SUMMARY

1. The clot accelerating effect of exposure of blood and plasma to glass surfaces was measured quantitatively, employing a two-stage procedure to measure residual prothrombin.

2. Prothrombin utilization in normal human blood was shown to be quantitatively and directly related to the degree of exposure of the blood to glass.

3. The prothrombin utilization defect of hemophilic blood was in some instances completely overcome by exposure of the blood to large glass surface area.

4. The prothrombin utilization defect of thrombocytopenic blood was invariably overcome by exposure of the blood to large glass surface area.

5. Prothrombin utilization in plasma from normal subjects was shown to be directly related to the platelet level and to the degree of exposure of the plasma to glass surface.

6. Prothrombin utilization did occur in "platelet-free" plasma and was directly related to the degree of exposure of the plasma to glass surface.

## ACKNOWLEDGMENT

The authors wish to express their appreciation to Mr. Alex Starr for his technical assistance in the performance of these experiments.

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# THE CEREBRAL CIRCULATION AND METABOLISM IN HYPERTHYROIDISM AND MYXEDEMA [1]

By WILLIS SENSENBACH, LEONARD MADISON, SEYMOUR EISENBERG, AND LAMAR OCHS

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(Submitted for publication May 27, 1954; accepted June 30, 1954)

*In vitro* studies (1–5) of the effect of variations in the functional activity of the thyroid gland upon cerebral metabolism have yielded conflicting results; some investigators have found the oxygen consumption of the brain to be increased in experimental hyperthyroidism, and diminished after thyroidectomy; others have not been able to demonstrate a change in the oxygen uptake of brain slices of either hyper- or hypothyroid animals. The introduction of the nitrous oxide method for the determination of cerebral blood flow provided a means of directly measuring cerebral circulatory and metabolic functions in man, and this technique was promptly applied to a study of these functions in human subjects with hyperthyroidism and myxedema. The initial studies in man, however, have also yielded conflicting results, especially with respect to the cerebral circulatory changes in thyroid disorders. Scheinberg (6), and Sokoloff, Wechsler, Balls, and Kety (7) found that the mean cerebral blood flow of a small series of subjects with hyperthyroidism was not significantly different from the mean CBF previously determined in a series of normal young men. Sokoloff, Wechsler, Mangold, Balls, and Kety (8) later reported that the CBF in hyperthyroidism was increased, but attributed this increase to anemia. Scheinberg, Stead, Brannon, and Warren (9) found the mean CBF in subjects with myxedema to be significantly lower than in young normal subjects, an observation in agreement with the findings of Himwich, Daly, Fazekas, and Herrlich (10) who studied the rate of blood flow in cretins by means of a thermostromuhr placed in an internal jugular vein. In 1951, Madison, Sensenbach, and Ochs (11) reported the results of a preliminary study of cerebral circulatory and metabolic functions in hyperthyroidism and myxedema, in which the values for these functions, obtained after euthyroidism had been achieved, served as controls for comparison with pre-treatment findings. Since then, the study has been enlarged to include observations in 22 subjects with hyperthyroidism and 11 with myxedema and forms the basis for the present report. Studies were repeated after euthyroidism had been achieved by treatment in 16 of the 22 hyperthyroid subjects and in 8 of the 11 subjects with myxedema.

## CLINICAL MATERIAL AND METHODS

Twenty-two males with hyperthyroidism whose ages ranged from 24 to 64 years were studied. The diagnosis was established when the characteristic history, physical findings, and laboratory studies clearly indicated the existence of a hypermetabolic state due to overactivity of the thyroid gland. Prior to the institution of definitive therapy, there was a period of observation during which the patients were treated with bed rest, hyperalimentation, supplementary vitamins, and sedation as needed. Just prior to the institution of treatment the initial circulatory studies were made; sedative drugs were withheld for at least 24 hours prior to the studies. Five of the patients were treated surgically, 14 with $I^{131}$ and 3 with propylthiouracil. Cerebral circulatory and metabolic studies were repeated in 16 of the subjects after a euthyroid state had been attained; in six subjects euthyroid studies were not done because of technical difficulties or failure of the patients to return to the hospital for follow-up examination.

Eleven male subjects with myxedema ranging in age from 24 to 68 years were studied. The etiology of the myxedema was post-thyroidectomy in five, post $I^{131}$ in one, spontaneous in four, and secondary to hypopituitarism in one. The cerebral circulatory studies were performed just prior to treatment and were repeated after the administration of thyroid substance had restored the euthyroid state.

The functional status of the thyroid gland was evaluated on the basis of clinical findings, BMR, radioactive

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TABLE I

*Cerebral circulatory and metabolic functions in hyperthyroidism, before and after treatment*

| Subject | Age | Treatment | BMR % | | $I^{131}$ Uptake % | | Mean arterial blood pressure MABP *mm. Hg* | | Cerebral blood flow CBF *cc./min./100 gm.* | | Cerebral vascular resistance CVR *mm. Hg/cc./min./100 gm.* | | Cerebral oxygen consumption $CMRO_2$ *cc./min./100 gm.* | | Cerebral glucose consumption $CMR_{glu}$ *mgm./min./100 gm.* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid |
| O. W. | 64 | $I^{131}$ and propyl. | +47 | +12 | 73 | | 79 | 96 | 72 | 60 | 1.10 | 1.60 | 3.63 | 3.47 | 2.9 | 4.2 |
| R. F. B. | 59 | $I^{131}$ and propyl. | +59 | + 7 | 67 | | 79 | 96 | 88 | 75 | 0.90 | 1.28 | 4.54 | 4.49 | 4.4 | 7.5 |
| G. E. | 59 | $I^{131}$ | +28 | + 7 | 66 | 33 | 85 | 97 | 85 | 44 | 1.00 | 2.20 | 3.96 | 2.60 | 4.3 | 2.6 |
| C. W. | 59 | $I^{131}$ | +23 | − 4 | 79 | 12 | 95 | 101 | 64 | 48 | 1.48 | 2.10 | 4.49 | 4.50 | 5.1 | 4.3 |
| R. K. | 56 | $I^{131}$ and propyl. | +60 | +13 | 62 | | 100 | 94 | 130 | 60 | 0.77 | 1.57 | 5.59 | 3.49 | 7.8 | 2.4 |
| R. H. | 56 | Propyl. | +39 | − 4 | | | 95 | 102 | 103 | 69 | 0.92 | 1.48 | 5.86 | 5.00 | 6.2 | 7.6 |
| J. R. | 56 | Propyl. and thyroidectomy | +39 | +16 | 54 | | 103 | 119 | 78 | 57 | 1.32 | 2.09 | 3.73 | 3.75 | | 7.4 |
| C. R. | 49 | Propyl. and thyroidectomy | +68 | +11 | 67 | 14 | 97 | 109 | 91 | 68 | 1.07 | 1.60 | 5.26 | 3.36 | 7.3 | |
| S. A. | 43 | $I^{131}$ | +38 | | 68 | | 81 | | 72 | | 1.13 | | 3.92 | | 4.3 | |
| H. E. | 41 | $I^{131}$ | +43 | | 67 | | 68 | | 73 | | 0.93 | | 4.41 | | 6.6 | |
| W. F. | 40 | $I^{131}$ | +67 | +16 | 73 | 42 | 76 | 84 | 70 | 53 | 1.09 | 1.58 | 4.00 | 3.61 | 5.1 | 6.4 |
| R. Y. | 37 | $I^{131}$ | +40 | + 9 | 74 | 5 | 96 | 91 | 68 | 56 | 1.41 | 1.63 | 4.70 | 4.00 | 2.0 | 5.6 |
| G. S. | 36 | $I^{131}$ | +48 | +10 | 64 | 17 | 80 | 87 | 93 | 77 | 0.86 | 1.13 | 3.96 | 4.76 | 4.7 | 4.8 |
| L. T. | 34 | $I^{131}$ and propyl. | +44 | | 67 | | 91 | | 120 | | 0.76 | | 7.12 | | 8.4 | |
| H. H. | 33 | Propyl. and thyroidectomy | +97 | + 5 | 58 | 16 | 78 | 81 | 63 | 55 | 1.24 | 1.47 | 3.87 | 3.94 | 5.0 | 2.8 |
| R. S. | 32 | $I^{131}$ | +32 | −12 | 80 | | 94 | 97 | 64 | 43 | 1.47 | 2.26 | 4.28 | 4.38 | 7.3 | 6.4 |
| E. M. | 31 | Propyl. | +44 | +11 | 60 | | 78 | 80 | 92 | 72 | 0.85 | 1.11 | 4.63 | 4.54 | 7.4 | 5.0 |
| J. B. | 31 | $I^{131}$ and propyl. | +90 | +19 | 79 | | 78 | 80 | 98 | 65 | 0.80 | 1.23 | 4.00 | 4.08 | 2.9 | 6.5 |
| L. S. | 31 | Thyroidectomy | +67 | − 4 | 77 | | 87 | 93 | 76 | 65 | 1.14 | 1.43 | 4.04 | 4.96 | 5.3 | |
| L. B. | 28 | $I^{131}$ | +53 | | 78 | | 93 | | 83 | | 1.12 | | 5.57 | | 3.3 | |
| R. L. S. | 26 | Propyl. | +80 | | 97 | | 84 | | 102 | | 0.82 | | 5.30 | | 5.1 | |
| R. B. | 24 | Thyroidectomy | +42 | | 64 | | 92 | | 114 | | 0.81 | | 5.85 | | 5.9 | |
| Mean | 42 | | +52 | + 5 | 70 | 20 | 87 | 94 | 86 | 60 | 1.05 | 1.61 | 4.67 | 4.06 | 5.3 | 5.3 |
| P value | | | | <.001 | | <.001 | | <.01 | | <.001 | | <.001 | | >.10 | | > .90 |

TABLE II

*Blood constituents in hyperthyroidism, before and after treatment*

| Subject | Age | Arterial oxygen content vol. % | | Cerebral venous oxygen content vol. % | | Arterial-cerebral venous oxygen difference vol. % | | Cerebral oxygen extraction ratio $ERO_2$ % | | Arterial carbon dioxide content vol. % | | Cerebral venous carbon dioxide content vol. % | | Arterial pH | | Hemoglobin gm. % | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid | Hyperthyroid | Euthyroid |
| O. W. | 64 | 13.60 | 15.97 | 8.56 | 10.19 | 5.04 | 5.78 | 37 | 36 | 52.27 | 51.70 | 56.73 | 56.63 | 7.41 | 7.37 | 11 | 12 |
| R. F. B. | 59 | 16.67 | 17.70 | 11.51 | 11.71 | 5.16 | 5.99 | 31 | 34 | 47.10 | 50.41 | 52.39 | 55.59 | 7.37 | 7.42 | 13 | 13 |
| G. E. | 59 | 19.29 | 21.07 | 14.63 | 15.16 | 4.66 | 5.91 | 24 | 28 | 51.09 | 43.82 | 56.21 | 49.10 | 7.36 | 7.44 | 15 | 15 |
| G. W. | 59 | 19.90 | 20.77 | 12.88 | 11.39 | 7.02 | 9.38 | 35 | 45 | 46.43 | 42.43 | 52.59 | 50.82 | 7.40 | 7.39 | 15 | 16 |
| R. K. | 56 | 15.89 | 18.00 | 11.59 | 12.18 | 4.30 | 5.82 | 27 | 32 | 47.81 | 45.31 | 52.00 | 50.78 | | 7.45 | 11 | |
| R. H. | 56 | 17.22 | 20.74 | 11.53 | 13.50 | 5.69 | 7.24 | 33 | 35 | 49.38 | 45.58 | 55.05 | 52.96 | 7.35 | 7.37 | 13 | 13 |
| J. R. | 56 | 17.83 | 18.69 | 13.05 | 12.99 | 4.78 | 5.70 | 27 | 30 | 53.68 | 53.44 | 58.55 | 59.14 | | | 14 | 13 |
| C. R. | 49 | 19.60 | 16.34 | 13.82 | 11.40 | 5.78 | 4.94 | 29 | 30 | 45.21 | 48.55 | 50.86 | 53.19 | | | 16 | 15 |
| S. A. | 43 | 16.42 | | 10.97 | | 5.45 | | 33 | | 46.42 | | 51.38 | | 7.37 | | 14 | |
| H. E. | 41 | 16.17 | | 10.13 | | 6.04 | | 37 | | 51.67 | | 57.92 | | 7.43 | | | |
| W. F. | 40 | 16.84 | 19.60 | 11.12 | 12.78 | 5.72 | 6.82 | 34 | 35 | 47.65 | 47.16 | 52.53 | 53.21 | | 7.39 | 12 | 14 |
| R. Y. | 37 | 20.53 | 21.87 | 13.62 | 14.72 | 6.91 | 7.15 | 34 | 33 | 48.96 | 44.93 | 56.54 | 51.83 | 7.41 | | 15 | 15 |
| G. S. | 36 | 14.96 | 19.49 | 10.70 | 13.28 | 4.26 | 6.18 | 28 | 32 | 52.18 | 46.95 | 55.59 | 52.84 | | | 13 | 14 |
| L. T. | 34 | 17.21 | | 11.28 | | 5.93 | | 34 | | 43.39 | | 48.73 | | 7.31 | | 15 | |
| H. H. | 33 | 18.07 | 18.20 | 11.93 | 11.03 | 6.14 | 7.17 | 34 | 39 | 39.04 | 45.06 | 45.27 | 51.88 | 7.35 | 7.36 | 15 | 14 |
| R. S. | 32 | 18.99 | 21.20 | 12.30 | 11.03 | 6.69 | 10.19 | 35 | 47 | 47.85 | 47.68 | 53.57 | 56.86 | | | 16 | 16 |
| E. M. | 31 | 17.85 | 18.08 | 12.82 | 11.77 | 5.03 | 6.31 | 28 | 35 | 49.42 | 46.17 | 54.67 | 52.42 | 7.41 | | 14 | 14 |
| J. B. | 31 | 16.75 | 19.81 | 12.67 | 13.53 | 4.09 | 6.28 | 24 | 32 | 53.44 | 42.23 | 57.74 | 49.68 | | | 14 | 16 |
| L. S. | 31 | 17.97 | 20.34 | 12.66 | 12.71 | 5.31 | 7.63 | 30 | 38 | 47.34 | 45.04 | 51.98 | 52.63 | | | 15 | 16 |
| L. B. | 28 | 21.17 | | 14.46 | | 6.71 | | 32 | | 48.79 | | 55.54 | | | | 15 | |
| R. L. S. | 26 | 17.17 | | 11.97 | | 5.20 | | 30 | | 47.48 | | 52.87 | | 7.37 | | 13 | |
| R. B. | 24 | 19.28 | | 14.15 | | 5.13 | | 27 | | 50.23 | | 54.38 | | | | 15 | |
| Mean | 42 | 17.70 | 19.24 | 12.20 | 12.46 | 5.50 | 6.78 | 31 | 35 | 48.49 | 46.65 | 53.78 | 53.10 | 7.38 | 7.40 | 14.0 | 14.4 |
| P value | | | <.01 | | >.8 | | <.001 | | <.001 | | >.05 | | >.4 | | >.10 | | >.9 |

TABLE III

*Cerebral circulatory and metabolic functions in myxedema, before and after treatment*

| Subject | Age | Diagnosis | BMR % | | $I^{131}$ Uptake % | | Mean arterial blood pressure MABP *mm. Hg* | | Cerebral blood flow CBF *cc./min./100 gm.* | | Cerebral vascular resistance CVR *mm. Hg/cc./100 gm./min.* | | Cerebral oxygen consumption $CMRO_2$ *cc./min./100 gm.* | | Cerebral glucose consumption $CMR_{glu}$ *mgm./min./100 gm.* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid |
| F. E. | 68 | Spontaneous myxedema | −21 | + 1 | 0.9 | | 95 | 87 | 46 | 51 | 2.07 | 1.71 | 4.23 | 3.32 | 5.1 | |
| J. S. | 61 | Spontaneous myxedema | −32 | + 4 | 1.3 | | 78 | 69 | 43 | 57 | 1.81 | 1.21 | 3.03 | 3.16 | 4.3 | 3.4 |
| B. H. | 56 | Pituitary myxedema | −27 | + 1 | 9.8 | | 62 | 62 | 46 | 56 | 1.35 | 1.11 | 3.60 | 3.17 | 5.5 | 5.6 |
| D. B. | 56 | Spontaneous myxedema | −26 | | 3.3 | | 92 | | 42 | | 2.19 | | 2.98 | | 3.8 | |
| A. B. B. | 55 | Postoperative myxedema | −28 | − 7 | 1.5 | | 109 | 90 | 31 | 50 | 3.52 | 1.80 | 2.64 | 3.99 | 3.7 | 7.5 |
| H. C. | 44 | Spontaneous myxedema | −29 | − 1 | 5.5 | | 80 | 63 | 40 | 55 | 2.00 | 1.15 | 2.84 | 3.90 | 2.4 | 4.4 |
| A. B. | 32 | Postoperative myxedema | −23 | | 5.0 | | 88 | | 58 | | 1.52 | | 4.30 | | 5.2 | |
| L. S. | 31 | Postoperative myxedema | −29 | + 1 | 5.0 | | 92 | 75 | 50 | 60 | 1.84 | 1.25 | 3.62 | 3.64 | 4.0 | 3.0 |
| J. B. | 31 | Post $I^{131}$ myxedema | −22 | +19 | 3.8 | | 68 | 80 | 49 | 65 | 1.39 | 1.23 | 3.61 | 4.08 | 5.4 | 6.5 |
| C. L. | 30 | Postoperative myxedema | −24 | + 2 | 2.0 | | 96 | 71 | 60 | 84 | 1.60 | 0.85 | 3.85 | 4.62 | 7.2 | 7.6 |
| R. B. | 24 | Postoperative myxedema | −31 | | 3.3 | | 97 | | 55 | | 1.76 | | 3.75 | | 5.8 | |
| Mean | 44 | | −27 | + 3 | 3.8 | | 87 | 75 | 47 | 60 | 1.91 | 1.29 | 3.50 | 3.74 | 4.8 | 5.4 |
| P value | | | | <.001 | | | | <.05 | | <.001 | | <.01 | | >.10 | | > .20 |

TABLE IV

*Blood constituents in myxedema, before and after treatment*

| Subject | Age | Arterial oxygen content vol. % | | Cerebral venous oxygen content vol. % | | Arterial-cerebral venous oxygen difference vol. % | | Cerebral oxygen extraction ratio $ERO_2$ % | | Arterial carbon dioxide content vol. % | | Cerebral venous carbon dioxide content vol. % | | Arterial pH | | Hemoglobin gm. % | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid | Euthyroid |
| F. E. | 68 | 18.07 | 17.05 | 8.88 | 10.55 | 9.19 | 6.50 | 51 | 38 | 46.76 | 50.33 | 54.23 | 56.24 | | | 14.0 | 13.0 |
| J. S. | 61 | 15.24 | 15.54 | 8.19 | 9.98 | 7.05 | 5.56 | 46 | 37 | 45.62 | 46.02 | 52.85 | 51.23 | | | 12.0 | 13.2 |
| B. H. | 56 | 14.74 | 14.63 | 6.91 | 8.97 | 7.83 | 5.66 | 53 | 39 | 45.54 | 51.36 | 54.49 | 57.97 | 7.41 | 7.42 | 11.2 | 10.5 |
| D. B. | 56 | 16.62 | | 9.52 | | 7.10 | | 43 | | 42.30 | | 50.06 | | 7.51 | | | |
| A. B. B. | 55 | 19.22 | 19.34 | 10.71 | 11.37 | 8.51 | 7.97 | 44 | 41 | 43.92 | 43.88 | 53.46 | 52.14 | | | 15.4 | 14.4 |
| H. C. | 44 | 13.83 | 16.89 | 6.74 | 9.80 | 7.09 | 7.09 | 51 | 42 | 47.97 | 43.44 | 54.42 | 49.45 | 7.37 | 7.33 | 10.6 | 13.7 |
| A. B. | 32 | 19.88 | | 12.47 | | 7.41 | | 37 | | 43.33 | | 50.90 | | 7.38 | | 15.5 | |
| L. S. | 31 | 19.34 | 19.40 | 12.11 | 13.33 | 7.23 | 6.07 | 37 | 31 | 50.95 | 49.05 | 57.27 | 55.18 | | | 15.0 | 15.8 |
| J. B. | 31 | 17.73 | 19.81 | 10.37 | 13.53 | 7.36 | 6.28 | 42 | 32 | 45.17 | 42.23 | 52.33 | 49.68 | 7.38 | | 15.8 | 15.5 |
| C. L. | 30 | 17.76 | 17.08 | 11.35 | 11.58 | 6.41 | 5.50 | 36 | 32 | 50.02 | 46.10 | 55.49 | 51.93 | 7.39 | | 13.7 | 12.8 |
| R. B. | 24 | 17.36 | | 10.54 | | 6.82 | | 39 | | 52.60 | | 58.83 | | | | 14.0 | |
| Mean | 44 | 17.25 | 17.47 | 9.80 | 11.14 | 7.45 | 6.33 | 44 | 37 | 46.74 | 46.55 | 54.03 | 52.98 | 7.41 | 7.38 | 13.7 | 13.6 |
| P value | | | >.30 | | <.01 | | <.01 | | <.001 | | >.7 | | >.2 | | | | >.8 |

iodine uptake, and serum protein bound iodine; subjects exhibiting borderline or equivocal findings were excluded from the study.

The cerebral blood flow (CBF) was determined by the nitrous oxide method of Kety and Schmidt (12), as modified by Scheinberg and Stead (13). Cerebral oxygen consumption ($CMRO_2$) was calculated from the CBF and the arterio-cerebral venous oxygen difference, cerebral glucose consumption ($CMR_{Glu}$) from the CBF and the arterio-cerebral venous glucose difference, and cerebral vascular resistance (CVR) from the CBF and the mean arterial blood pressure (MABP). MABP was measured directly from a peripheral artery with a damped mercury manometer. Blood oxygen content and carbon dioxide content were determined manometrically. Blood sugar was determined by Nelson's modification of the Somogyi method (14). Blood samples for pH determination were drawn anerobically and the pH immediately determined at 37° by means of a Cambridge Research Model pH Meter. The cerebral blood flow determinations were done on recumbent subjects in the post-absorptive state.

The data were analyzed statistically by the method of paired observations; the pertinent difference between values obtained before and after treatment were calculated for each subject. The mean of these differences together with its standard error was then calculated from the individual differences. P values so determined are recorded in Tables I to IV.

## RESULTS

### *Hyperthyroidism*

The results of cerebral circulatory and metabolic studies in 22 subjects with hyperthyroidism are summarized in Tables I and II. These tables also include results obtained in 16 of 22 subjects when the studies were repeated after successful treatment of the hyperthyroidism. Comparison of the pre-treatment observations with control or euthyroid values reveals that hyperthyroidism is associated with a 42 per cent increase in CBF (60 to 86 cc. per min. per 100 gm.) ($P = < .001$); a 35 per cent decrease in CVR (1.61 to 1.05 mm. Hg per cc. per min. per 100 gm.) ($P = < .001$), and a 7 per cent decrease in MABP (94 to 87 mm. Hg) ($P = < .01$). These circulatory changes, however, are not accompanied by changes in cerebral oxygen utilization, for the increase in CBF is associated with a decrease in cerebral $O_2$ extraction (35 to 31 per cent) ($P = < .001$) and a decline in arteriocerebral venous oxygen difference (6.78 to 5.50 vols. per cent) ($P = < .001$). Cerebral glucose consumption is likewise unchanged. The only significant change observed in the blood constituents measured is the decline in arterial $O_2$ content (19.24 to 17.70 vols. per cent) ($P = < .01$). This change may be related to the alterations in pulmonary ventilation and gas exchange which occur in hyperkinetic states (15, 16).

### *Myxedema*

The results of studies in 11 subjects with myxedema, and the findings obtained in 8 of these subjects after a euthyroid state had been established by adequate substitution therapy are summarized in Tables III and IV. These data reveal changes in cerebral hemodynamics opposite to those observed in hyperthyroidism. There is a 22 per cent reduction in CBF (60 to 47 cc. per min. per 100 gm.) ($P = < .001$), a 48 per cent increase in CVR (1.29 to 1.91 mm. Hg per cc. per min. per 100 gm.) ($P = < .01$), and a 16 per cent increase in MABP (75 to 87 mm. Hg) ($P = < .05$) in myxedema, as compared to the euthyroid control values in these subjects.

As in the case of hyperthyroidism, however, the circulatory changes are not associated with changes in the rate of cerebral oxygen utilization, the reduction in CBF being accompanied by increased cerebral oxygen extraction (19 per cent) ($P = < .001$) and an increase in arterio-cerebral venous oxygen difference (6.33 to 7.45 vols. per cent) ($P = < .01$). Cerebral glucose consumption is also unaltered. Reduction in cerebral venous oxygen content (11.41 to 9.80 vols. per cent) ($P = < .01$) is the only significant change to occur in the blood constituents.

The difference in mean $CMRO_2$ between euthyroid subjects who had been treated for hyperthyroidism (4.06 cc. per min. per 100 gm.) and those who had been treated for hypothyroidism (3.74 cc. per min. per 100 gm.) is not statistically significant ($P = > .2$).

## DISCUSSION

These studies indicate that the cerebral circulation shares in the over-all hemodynamic alterations that occur as a consequence of an excess or deficiency of the thyroid hormone. They provide no evidence to indicate that the thyroid hormone exerts a specific effect upon the cerebral circulation, for the changes occur in the absence of alterations in the constituents of the blood that are known to be of importance in the specific regu-

lation of the cerebral vascular tone and of cerebral blood flow. They are, rather, changes which quantitatively and qualitatively parallel the variations in cardiac output and total peripheral vascular resistance that accompany hyperthyroidism and myxedema. Nor does it appear that the thyroid hormone exerts an influence upon the rate of cerebral oxygen or glucose utilization, for the data show that these functions are normal in both hyperthyroidism and myxedema, and they remain unchanged when euthyroidism is restored by appropriate treatment. This failure to demonstrate a relationship between blood flow and metabolic activity of the brain casts doubt, as Scheinberg (6) has previously indicated, upon the generally accepted concept that changes in the general circulation in thyroid disease occur in response to alterations in metabolic demands of the tissues. It indicates rather, at least as far as the brain is concerned, that they occur as a simple consequence of an effect of the thyroid hormone upon the heart and peripheral blood vessels.

The discrepancies which exist between the results of this investigation and the cerebral circulatory changes reported by others in hyperthyroidism are probably best accounted for by the different means by which these studies were controlled. Scheinberg (6) and Sokoloff, Wechsler, Balls, and Kety (7) compared the cerebral circulatory and metabolic functions in a small series of patients with hyperthyroidism with mean values for these functions previously determined in normal young males. In the present study and in the report by Sokoloff, Wechsler, Mangold, Balls, and Kety (8) a more valid basis for comparison of the results was obtained by repeating the measurements after euthyroidism had been achieved by treatment. Although Scheinberg (6) found the mean CBF in hyperthyroidism to be unchanged as compared with normal young men, the mean arterio-cerebral venous oxygen difference of his subjects was 5.50 vols. per cent, a value identical to that of this present study. Scheinberg attributed this low A–V oxygen difference to extracerebral contamination of cerebral venous blood but suggested that further study might reveal it to be a reflection of increased CBF. When Sokoloff, Wechsler, Mangold, Balls, and Kety (8) later employed the values of post-treatment studies as controls, they found the CBF in hyperthyroidism to be increased, but the decline in CBF which they observed after treatment was not statistically significant, and they attributed the increase in CBF to the presence of anemia in their subjects. However, a review of their data casts doubt upon the validity of their "controls." Three of the seven post-treatment studies were done when the BMR was + 25 per cent or more; in one instance there was no change in BMR (+ 30 to + 28 per cent) after treatment; and in another the BMR was − 35 per cent when the control studies were done. It is unlikely, therefore, that these values are accurately representative of the "normal" or euthyroid state.

The increased CVR and reduced CBF in myxedema is in agreement with the results of Himwich, Daly, Fazekas, and Herrlich (10) in cretins and of Scheinberg, Stead, Brannon, and Warren (9) in adult hypothyroidism. In contrast to the present studies, however, these investigators found evidence of depressed cerebral metabolism in hypothyroidism. Himwich, Daly, Fazekas, and Herrlich observed that the treatment of cretinism was accompanied by an increase in CBF and a decrease in arterio-cerebral venous oxygen difference. They "corrected" the measured fall in arteriocerebral venous oxygen difference for the observed increase in CBF and in this manner estimated that the rate of cerebral oxygen uptake was increased after the institution of treatment. Our findings in adult hypothyroidism indicate that this "correction" is invalid, for the arterio-cerebral venous oxygen difference after treatment changes in a reciprocal fashion to the changes in CBF, cerebral $O_2$ utilization remaining unaltered. Scheinberg, Stead, Brannon, and Warren (9), using methods similar to our own, also found evidence of depression of cerebral metabolism in myxedema. The mean arterio-cerebral venous oxygen difference reported by these authors (6.48 vols. per cent) did not significantly differ from their normal subjects. However, when their data are recalculated and four observations that were made 6 to 15 months after treatment are excluded, a mean arterio-cerebral venous oxygen difference of 7.23 vols. per cent is obtained; a value which closely approximates the significantly increased mean arterio-cerebral venous oxygen difference (7.45 vols. per cent) found in untreated myxedema in the present study.

The absence of change in cerebral metabolism following the successful treatment of either hypo- or hyperthyroidism, and the fact that the rate of cerebral oxygen and glucose uptake in subjects made euthyroid after the treatment of hyperthyroidism does not significantly differ from the rate of uptake of these substances in subjects made euthyroid after treatment of myxedema, in our opinion, constitutes strong evidence that the rate of cerebral metabolism is uninfluenced by the thyroid hormone.

## CONCLUSIONS

1. Cerebral circulatory and metabolic functions have been measured before and after treatment in 16 of 22 subjects with hyperthyroidism and in 8 of 11 subjects with myxedema. Hyperthyroidism was found to be accompanied by diminished cerebral vascular resistance and increased blood flow; myxedema by increased cerebral vascular resistance and reduced blood flow. The cerebral circulation is restored to normal in both instances when euthyroidism is achieved by appropriate treatment.

2. The rate of cerebral oxygen consumption and glucose consumption is normal in hyperthyroidism and myxedema and is unaltered by the treatment of either.

3. The cerebral circulation apparently shares equally in the general circulatory changes incident to alterations in thyroid function. Oxygen and glucose consumption of the brain is not influenced by thyroid hormone.

## ACKNOWLEDGMENTS

This work was done with the technical assistance of Mrs. Angeline M. Hayes and Miss Mildred T. Brennan. Mr. Tyree Loving and Mr. W. R. Hampton also gave valuable assistance.

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# OBSERVATIONS ON THE SODIUM-RETAINING CORTICOID (ALDOSTERONE) IN THE URINE OF CHILDREN AND ADULTS IN RELATION TO SODIUM BALANCE AND EDEMA[1]

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(Submitted for publication June 9, 1954; accepted July 7, 1954)

Patients with lipemic nephrosis, cardiac failure, and hepatic cirrhosis frequently accumulate an excess of extracellular fluid, distributed in a pattern characteristic of the underlying disease. These patients commonly have a marked impairment of sodium excretion, while glomerular filtration may be low, normal, or even increased (1–4). This impairment of sodium excretion suggested the presence of a stimulus to tubular reabsorption of sodium, such as a sodium-retaining hormone with an action like desoxycorticosterone. Since the chemical nature of the hormone was unknown, a biologic assay was used to measure the sodium-retaining activity of lipid extracts of human urine.

Unusually high sodium-retaining activity was present in the urine of some edematous patients with cardiac and renal disease (5). In lipemic nephrosis, this increased sodium-retaining activity was reduced when diuresis followed the use of cortisone, corticotrophin, or concentrated human serum albumin (6, 7). Other observers, using similar methods, have also found abnormal sodium-retaining activity in the urine of patients with nephrosis, heart failure, cirrhosis, and toxemia of pregnancy with edema (8–12).

When extracts of urine from patients with lipemic nephrosis were fractionated by chromatography, the sodium-retaining activity was found to be more polar than desoxycorticosterone (13) and to be concentrated in one fraction which closely resembles aldosterone (14–16).

The present report summarizes our results with material extracted by chloroform within 40 minutes after acidification of urine to pH 1.0. Although this readily hydrolyzed and extracted material does not include all of the daily output of sodium-retaining hormone, it contains a reproducible fraction of the output. The results are comparable to those obtained in other laboratories, and can be used as a basis for examination of several questions which have arisen. For example, what is the relation of the output of sodium-retaining activity to the presence of edema and to the output of sodium and water? Does the sodium-retaining material excreted in several diseases have the same chromatographic and physiologic properties? Some data related to these questions will be presented here.

## METHODS

Urine was collected as 24-hour specimens in a refrigerator without preservative. Sodium was determined by flame photometer. Each day's total specimen was promptly frozen and held at $-20°$ C. until extraction.

Urine was acidified to pH 1.0 with concentrated HCl and was shaken with four successive portions of chloroform (not less than 100 cc. and at least 0.1 volume per volume of urine). Emulsions were broken by centrifugation. The extraction was completed within 40 minutes after the urine was acidified. The chloroform extracts were combined, washed with cold 0.1 N sodium hydroxide solution and cold distilled water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure at 40° C. The residue was taken up in redistilled ethanol.

Sodium-retaining activity was determined by bioassay (17). The results are expressed as the dose of desoxycorticosterone acetate (DCA) which would produce an equivalent effect on the excretion of sodium and potassium by adrenalectomized rats. The amount of extract given to each rat is expressed in minutes (*e.g.*, 20 minutes = 1/72 of the extract from a 24-hour specimen).

In certain instances, urine extracts increase the excretion of sodium by the adrenalectomized rats. When this result is expressed as a dose of DCA, an estimate

[1] This project was supported by a research grant (A-119) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

less than zero (minus sign) is obtained. A similar result is seen when cortisone, hydrocortisone, or corticosterone is tested in the bioassay (17).

The present results on normal urine show sodium-retaining activity less than that found in earlier studies (18), in which the small but definite sodium-retaining effect of the injected solvent was overlooked and in which a less satisfactory method of estimating dosage was used.

## RESULTS

*Normal adults on unrestricted diets:* Eleven healthy subjects, whose ages ranged from 22 to 40 years, showed sodium-retaining activity between + 0.5 and − 7.2 (average − 2.8) μg. DCA per 20 minutes of extract (Figure 1).

*Normal children:* Five subjects, aged 3 to 11 years, on free sodium intake, showed sodium-retaining activity between + 0.5 and − 0.5 (average 0.0) μg. DCA per 20 minutes of extract.

*Restriction of sodium intake* to 11 mEq. per day was followed by a systematic increase in sodium-retaining activity in six normal adults (Table I). A simple sign test suffices to demonstrate that the increases in the series reflect real changes (at the 5 per cent level of significance) rather than random fluctuation.[2]

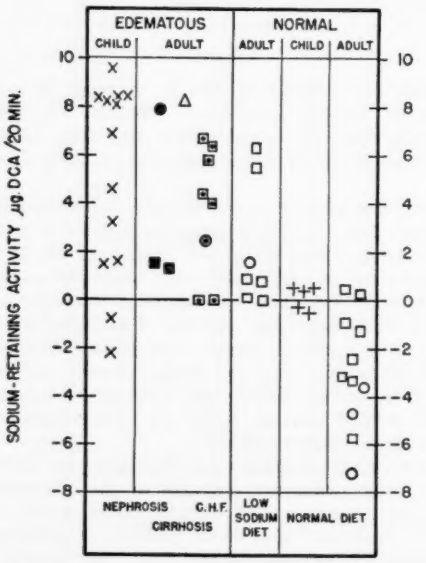


FIG. 1. SODIUM-RETAINING ACTIVITY OF URINE EXTRACTS

Symbols: Normal adults: male □, female ○; Congestive heart failure (C.H.F.): male ⊡, female ⊙; Adults with lipemic nephrosis: male ■, female ●; Children: normal +, lipemic nephrosis ×.

TABLE I

*Effect of reduced sodium intake (11 mEq. per day) on sodium-retaining activity of urine extracts of six normal adults*

| Patient | Sodium-retaining activity: Normal diet μg. DCA/20 min. | Sodium-retaining activity: Low sodium diet μg. DCA/20 min. | Change |
|---|---|---|---|
| He | +0.5 | +6.3 | +5.8 |
| Wo | +0.3 | +5.5 | +5.2 |
| Dj | −0.9 | 0.0 | +0.9 |
| Jl | −2.4 | +0.9 | +3.3 |
| Sp | −4.7 | +1.6 | +6.3 |
| Sa | −5.7 | +0.8 | +6.5 |
| Mean | −2.15 | +2.52 | +4.67 |

*Adults with edema due to heart failure, lipemic nephrosis, and hepatic cirrhosis:* Eight patients with congestive heart failure and edema showed sodium-retaining activity of 0 to 6.7 (average 3.7) μg. DCA per 20 minutes of extract. Three edematous adults with lipemic nephrosis showed variably increased sodium-retaining activity. One patient with Laennec's cirrhosis was studied to relate the present findings with previous observations (10); sodium-retaining activity was 8.2 μg. DCA per 20 minutes. The diets of these patients contained 10 to 85 mEq. sodium per day. There was no obvious relationship between sodium intake and sodium-retaining activity in this small, heterogeneous group.

*Children with edema due to lipemic nephrosis:* Thirteen patients showed a range of sodium-retaining activity between − 2.2 and + 9.6 (average 4.9) μg. DCA per 20 minutes of extract. The diets contained 10 to 51 mEq. sodium per day. Sodium-retaining activity showed no significant change when a free sodium intake was given to two patients.

*Relation of sodium-retaining activity to renal excretion of sodium:* In Figure 2, sodium-retain-

[2] More sensitive tests are possible. Each of the twelve determinations has a standard error of approximately 2.0 μg., since all assays involved eight or nine rats. It follows that the standard error of each difference is approximately 2.8 μg., and that the standard error of the average of six such differences is approximately $\frac{2.8}{\sqrt{6}} \doteqdot$ 1.15 μg. The average of the difference is 4.67 μg., more than three times its standard error, and thus highly significant.

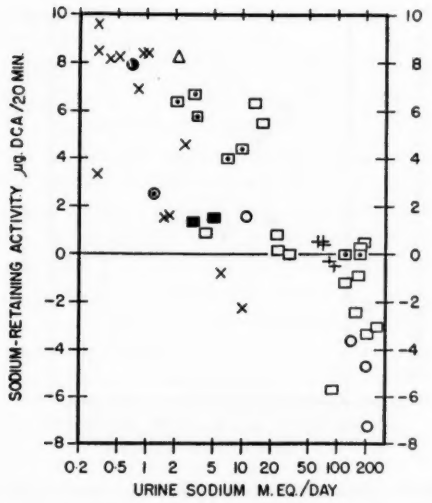


FIG. 2. RELATION BETWEEN URINE SODIUM AND SODIUM-RETAINING ACTIVITY

Assays and symbols are the same as in Figure 1. The logarithm of urine sodium is used empirically: compare the linear response and uniform variance of $\sqrt{Na}$ or log K/Na with dose of desoxycorticosterone in adrenalectomized rats (17).

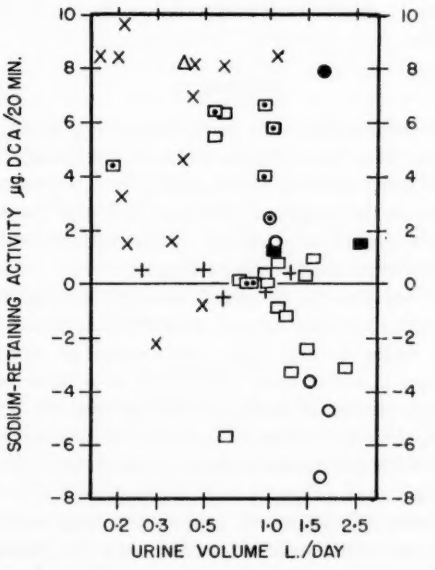


FIG. 3. ABSENCE OF CLEAR RELATION BETWEEN URINE VOLUME AND SODIUM-RETAINING ACTIVITY

Symbols in this and subsequent figures are the same as those used in Figure 1.

ing activity is plotted against the sodium content of the 24-hour specimen from which the extract was made. The assays and symbols for diagnoses are the same as in Figure 1. The inverse correlation of sodium-retaining activity with sodium output is clearly evident. It is apparent that the relationship varies somewhat in different patients, as might be expected with variations in the tubular load of sodium and in the excretion and estimation of sodium-retaining activity.

*Relation to urine volume:* The output of sodium-retaining activity appears to be independent of urine flow (Figure 3).

*Chromatographic behavior of the sodium-retaining corticoid:* When urine extracts from children with nephrosis were chromatographed on paper, sodium-retaining activity was concentrated in a fraction which moved near cortisone in the toluene and propylene glycol system (14). In the benzene and aqueous methanol system, the activity was associated with a corticosteroid whose lower mobility allowed its separation from cortisone. The chromatographic behavior of this

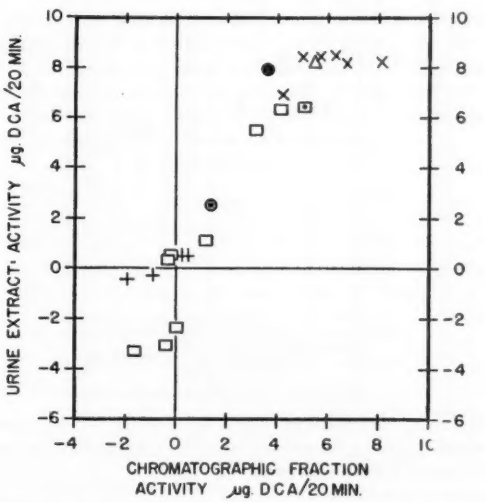


FIG. 4. COMPARISON OF SODIUM-RETAINING ACTIVITY OF EACH URINE EXTRACT (ORDINATE) WITH ACTIVITY OF THE "E FRACTION" FROM TOLUENE-PROPYLENE GLYCOL CHROMATOGRAM OF THAT EXTRACT (ABSCISSA)

The points each represent one extract; they approach, but do not reach, the 45° line ($x = y$), which would indicate complete recovery of the sodium-retaining activity of the extract in this single fraction of the chromatogram.

sodium-retaining corticoid corresponds to that described for aldosterone (19).

In Figure 4, the sodium-retaining activity of this chromatographic fraction is compared with the activity of the whole extract from which the fraction was prepared. Approximately 75 per cent of the activity of the whole extract was recovered in this fraction in the 22 cases studied. Significant sodium-retaining activity has not been found in any other chromatographic fraction.[3]

Figure 5 shows that sodium-retaining activity may appear in this fraction in nephrosis, in congestive heart failure, and in hepatic cirrhosis. Bioassay of a comparable fraction from the urine of six adults and four children on a free sodium intake showed no sodium-retaining activity in doses equivalent to 20 to 167 minutes of extract. The restriction of sodium intake to 11 mEq. per

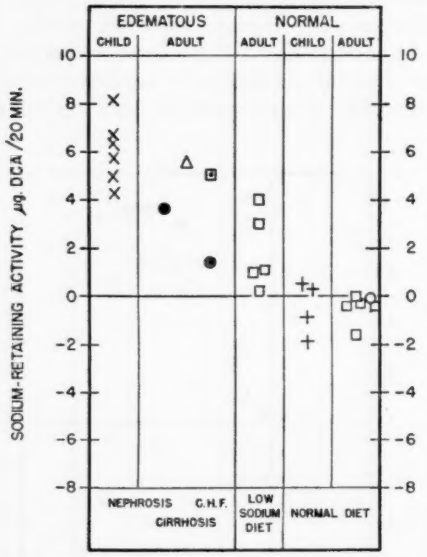


FIG. 5. SODIUM-RETAINING ACTIVITY OF "E FRACTION" FROM TOLUENE-PROPYLENE GLYCOL CHROMATOGRAMS OF VARIOUS URINE EXTRACTS

[3] A material which increases the output of sodium in the bioassay has been observed in the hydrocortisone fraction (14). When this material was eliminated by chromatography from the fraction in which sodium-retaining activity was sought, the latter fraction did not gain in sodium-retaining activity, as if an interfering substance had been removed; but the diuretic activity found in the urine extracts of some normal adults was reduced or eliminated (Figure 4).

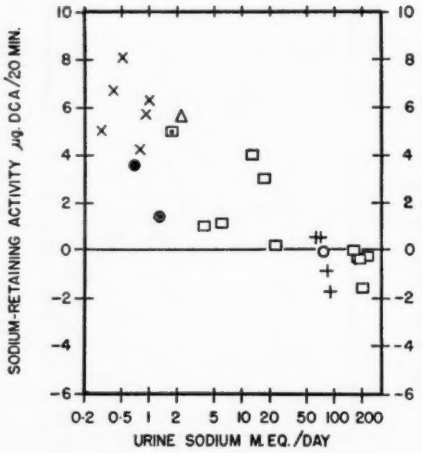


FIG. 6. RELATION OF URINE SODIUM AND SODIUM-RETAINING ACTIVITY OF "E FRACTION" OF TOLUENE-PROPYLENE GLYCOL CHROMATOGRAM OF URINE EXTRACT

day may be followed by the appearance of appreciable sodium-retaining activity in this chromatographic fraction (Figure 5).

The relationship between sodium output and the sodium-retaining activity of this fraction separated by paper chromatography is shown in Figure 6.

## DISCUSSION

If the bioassay of a sodium-retaining hormone in the urine measures an effective mechanism for conserving sodium, there should be a correlation between the output of this hormone and the patient's output of sodium. The data presented demonstrate such a relationship.

When greatly increased amounts of sodium-retaining hormone appear, accumulation of sodium and water in the body may result in edema. Edema is not of itself necessarily associated with a high output of sodium-retaining activity, however, since the output of sodium-retaining activity falls with increasing sodium output before edema is eliminated (Figures 1, 2; References 6, 7).

Increased output of sodium-retaining activity may be stimulated in normal men by reduced sodium in the diet. In this case, urine sodium is low, while a very moderate reduction of extracellular volume exists. It would appear that bioassay of sodium-retaining activity measures a

mechanism for the conservation of sodium independent of the state of hydration.

Sodium-retaining activity can be recovered in a single chromatographic fraction of urine extract. The same fraction contains the activity observed in lipemic nephrosis, in cardiac failure, and in hepatic cirrhosis, as well as in normal men on a diet low in sodium. In children with lipemic nephrosis, this active fraction has been found to contain a corticosteroid resembling aldosterone in a number of physical and chemical properties and in its ability to increase sodium reabsorption by the renal tubules (14, 15).

The failure to demonstrate sodium-retaining activity in extracts of normal human urine indicates only that readily hydrolyzed complexes are present in quantities too small to assay. More prolonged hydrolysis at pH 1.0 releases assayable quantities of sodium-retaining hormone from normal urine (20). Since this normal output disappears in patients subjected to adrenalectomy (20), it seems highly probable that the sodium-retaining hormone arises in the adrenal cortex.

The output of sodium-retaining hormone does not appear to be under the control of pituitary corticotrophin (21). The stimulus to increased output would appear best defined by an "inadequacy" of the circulation, including depletion of plasma or extracellular volume, as suggested by Peters (22). Since a number of the patients studied were not able to accept a full normal diet, it is possible that sodium intake was subnormal even when there was no deliberate restriction. Present experience indicates that the increased sodium-retaining activity in disease is not dependent upon low sodium intake alone, although reduced sodium intake may further increase the high levels observed.

## SUMMARY

Forty-eight specimens of urine from normal adults and children and from hospitalized patients have been extracted with chloroform within 40 minutes after acidification to pH 1.0. The sodium-retaining activity of the extracted material has been measured by bioassay. Significant sodium-retaining activity was observed in extracts prepared from the urine of a number of edematous patients with lipemic nephrosis, cardiac failure, and hepatic cirrhosis.

Urine extracts from normal men and women often had an opposite effect, tending to increase the excretion of sodium in the adrenalectomized rats, resembling the effect of hydrocortisone in the bioassay used.

Increased sodium-retaining activity was found in the urine of some normal men and women when sodium intake was reduced to 11 mEq. per day.

The level of sodium-retaining activity appeared to be related to the daily output of sodium by the patient, rather than to a specific disease, to the state of hydration, or to urine flow.

The sodium-retaining activity of extracts of urine from several patients with lipemic nephrosis, cardiac failure, or hepatic cirrhosis was found in the same chromatographic fraction of each extract tested. Some normal men on low sodium diets showed increased activity in the same fraction of the chromatograms. This fraction has been observed to contain a corticosteroid which is presumed to be aldosterone on the basis of its chromatographic and biologic properties.

## ACKNOWLEDGMENTS

The authors wish to thank Dr. B. J. Axelrad for performing several of the bioassays reported here, and Dr. L. E. Moses for advice on statistical methods. The assistance of the medical, nursing, and dietetic staffs of Stanford University Hospitals is gratefully acknowledged.

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# MEASUREMENT OF EXTRACELLULAR FLUID VOLUME IN NEPHRECTOMIZED DOGS [1]

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(Submitted for publication June 4, 1954; accepted July 9, 1954)

The volumes of distribution of a number of substances of widely varying molecular size and chemical properties, including sulfate, thiosulfate, mannitol, sucrose, and inulin, have been found to represent 15 to 25 per cent of body weight of man, dog, and other mammals. The apparently equal volumes of distribution of these dissimilar substances has suggested that the distribution of each of these substances measures the same portion of body fluid and has led to efforts to identify this volume of body fluid with the extracellular fluid volume.

In a series of individuals, however, there is a considerable range in the fraction of body weight which the volume of distribution of any one of these substances represents. In only a few instances have the distributions of two of these substances been compared simultaneously in the same individual. Schwartz (1) reported a ratio of thiosulfate volume to simultaneously measured mannitol volume of 0.90 (0.87 to 0.96) in four normal dogs, a ratio of 1.02 and 0.98 in two normal human subjects, and a ratio of inulin to simultaneously measured mannitol volume of 0.97 (0.93 to 1.02) in six normal human subjects (2). Walser, Seldin, and Grollman (3) reported a ratio of radiosulfate volume to simultaneously measured inulin volume of 0.95 (S.D. ± 0.11) in nine normal human subjects. Deane, Schreiner, and Robertson (4) measured volumes of sucrose and inulin in each of four normal human subjects on separate occasions and found an average ratio of sucrose volume to inulin volume of 0.97 (0.94 to 1.03). Most of these comparisons depend on quantitative recovery in the urine of infused inulin.

Kruhøffer's (5) data indicate a larger volume of distribution for sucrose than inulin in nephrectomized rabbits. Raisz, Young, and Stinson (6) have reported a ratio of inulin volume to thiosulfate volume of 0.76 (0.74 to 0.79) in four normal dogs and of sucrose volume to thiosulfate volume of 0.86 (0.86 to 0.87) in three normal dogs in which distributions of these pairs of substances were studied simultaneously. Nichols, Nichols, Weil, and Wallace (7) found that the ratio of inulin volume to thiosulfate volume averages 0.79 in five nephrectomized dogs.

To determine which, if any, of these substances distribute in equal volumes, the distributions of six substances: radiosulfate, thiosulfate, mannitol, sucrose, raffinose, and inulin, ranging in molecular weight from 96 to 5100 and including two anions, have been compared in nephrectomized dogs. These comparisons have been made under conditions which eliminate or minimize uncertainties regarding: a) extrarenal removal of these substances, b) changing plasma blank, c) completeness of recovery of the injected substance in the urine, and d) upper urinary tract dead space. The volumes of distribution of three of these substances, radiosulfate, thiosulfate, and mannitol, have been found to be equal. Sucrose, raffinose, and inulin volumes are smaller in inverse order to the molecular size of these substances.

Volumes of distribution of d-galactose, d-xylose, and l-arabinose have been reported to increase in nephrectomized dogs and rabbits after insulin administration, presumably the result of insulin facilitating transport of these substances across cell membranes (8, 9). The possibility that endogenous insulin release might alter volumes of distribution of the six substances studied has been investigated by observing the effect of intravenous insulin on their volumes of distribution.

## METHODS

### *A. Experimental plan*

1. In the analytical methods used for inulin, raffinose, and sucrose, these substances are mutually interfering. Therefore, the volumes of distribution of the six substances were compared in three groups of four or five

[1] This work was supported by a grant from the American Heart Association.

experiments each: *a*) A group in which inulin, mannitol, thiosulfate, and radiosulfate were infused; *b*) a group in which raffinose, mannitol, thiosulfate, and radiosulfate were infused; and *c*) a group in which sucrose, mannitol, thiosulfate, and radiosulfate were infused. In three control experiments sodium chloride solution equal in volume and approximately isosmotic with the solutions given in each of the first three groups of experiments was infused and changes in plasma blank for each of the six substances were determined under comparable experimental conditions.

2. The effect of insulin on the distribution of these six substances was determined in two experiments for each of the six substances. 0.2 units of crystalline zinc insulin per kilogram of body weight was injected intravenously four hours after infusion of these substances.

3. The distribution of inulin and radiochloride were determined simultaneously, as part of a separate study, under conditions essentially the same as those of the above experiments.

*B. Experimental procedure*

1. Healthy adult male dogs were anesthetized with sodium pentobarbital, weighed, and nephrectomized. Bilateral nephrectomy was accomplished through six cm. flank incisions by ligating capsular vessels, doubly ligating ureters, major branches of renal arteries and renal veins, and finally placing ligatures about the entire pedicles. The kidneys were left *in situ* and muscle and skin layers approximated with sutures. Following operation animals were kept lightly anesthetized. Adequacy of ligation was verified by autopsy following each experiment.

2. Polyethylene tubing was introduced into an iliac vein or inferior vena cava through the femoral vein and connected to a 50 cc. burette. Substances to be infused were transferred quantitatively to the burette, following which burette and tubing were washed with 3 per cent glucose. At 30-minute intervals for two hours after infusion and at hourly intervals for four additional hours, 20 cc. arterial blood samples were drawn into heparinized syringes. Plasma was promptly separated.

Ampouled inulin (U. S. Standard Products Co., lot 2-110A1) was heated in boiling water for 20 minutes, cooled and diluted to 100 cc. after adding 0.9 gm. NaCl. Fifty cc., containing approximately 2.2 gm. of inulin, were transferred to the infusion burette. An aliquot of the remainder was appropriately diluted for analysis. Quantities of raffinose, sucrose, and mannitol to give plasma concentrations of 75 to 100 mg. per 100 cc. were accurately weighed out shortly before infusion and dissolved separately in distilled water to make slightly hypertonic solutions. These were quantitatively transferred to the infusion burette. At the same time, and for each experiment, a sample of each substance to be infused was weighed out and diluted to volume to serve as a standard. A volume of 2.5 per cent hydrated sodium thiosulfate calculated to give an initial plasma concentration of 35 to 45 mg. per 100 ml. was pipetted into the infusion burette. An aliquot of the same solution was titrated with standard $KIO_3$ solution. Radiosulfate, obtained from Oak Ridge National Laboratory as carrier-free $H_2S^{35}O_4$, was appropriately diluted in isotonic saline and sulfuric acid added to give a concentration of 0.001 N sulfate in the solution infused. From 4 to 15 ml. of this solution, approximately 2.5 microcuries per kilogram of body weight, were infused $Cl^{36}$, obtained from Oak Ridge National Laboratory in 0.1 to 0.2 N HCl, was neutralized and diluted to 0.5 microcuries per ml. Approximately 0.25 microcuries per kilogram of body weight were infused.

In the first three groups of experiments, the four substances were infused in sequence over a 15-minute period. The mid-point of the thiosulfate infusion, the third substance infused, was taken as zero time. The infusions are estimated to have increased extracellular fluid by 2 to 4 per cent of pre-infusion volume.

3. Plasma volume was estimated two and six hours after infusion by dilution of T-1824 dye using the procedure described by Chinard (10). The mean of the two determinations was used in the calculations.

*C. Analytical methods*

Inulin concentration in aliquots of infusion fluid and in Somogyi filtrates of plasma was determined after 30-minute exposure to yeast by the method of Harrison (11). Dilutions of a solution of an accurately weighted sample of Difco inulin served as standards.

Plasma glucose was determined by the method of Nelson (12). Raffinose in an aliquot of the same filtrate was hydrolyzed in 0.1 N HCl at 80° C. for 25 minutes. After neutralization with NaOH, reducing sugar was determined by the method used for glucose. Raffinose concentration was calculated from the difference in raffinose equivalents of reducing substances present before and after acid hydrolysis. Acid hydrolysis did not increase the apparent glucose concentration of plasma not containing raffinose.

Sucrose was determined in cadmium filtrates by method of Schreiner (13), the optimal conditions for hydrolysis and color development described for inulin determination having been found to apply equally well for sucrose. Mannitol was determined in cadmium filtrates by the method of Corcoran and Page (14). In experiments in which sucrose was infused, sucrose and mannitol were determined in the same filtrates.

Plasma blanks of inulin, sucrose, and mannitol were determined additively.

Thiosulfate was determined by the indirect method of Newman, Bordley, and Winternitz (15). Concentrations are expressed as mg. per cent sodium thiosulfate.

Sulfate was precipitated by addition of $BaCl_2$ to trichloracetic acid filtrates of plasma and standards at 90° C. after addition of sufficient carrier sulfate to give a density thickness of 25 mg. per cm.$^2$ when the precipitate was transferred with 95 per cent alcohol to No. 42 Whatman, paraffin-rimmed filter discs. Benzidine was not used to precipitate sulfate since it also precipitates thiosulfate. Each duplicate sample was counted in a flow counter for a total of 10,000 counts at a counting rate of approxi-

mately 1,000 counts per min. with a background of approximately 20 counts per min.

Except for inulin, raffinose, and sucrose, which are mutually interfering, recoveries from plasma of each of the test substances in the presence of the others were satisfactory (96 to 104 per cent).

$Cl^{36}$ in plasma and standards was determined after digestion in concentrated nitric acid containing excess $AgNO_3$. The AgCl precipitate was transferred in glycerine-alcohol washes to No. 1 Whatman paraffin-rimmed filter discs. Each duplicate sample was counted with a 1.5 mg. per $cm.^2$ mica end window Geiger tube for a total of 10,000 counts at a counting rate of approximately 500 counts per min. and a background of approximately 18 counts per min. Variations in plasma chloride concentration encountered in these experiments do not introduce significant error.

Plasma and whole blood chloride concentrations were determined by the method of Van Slyke and Hiller (16). Hematocrits were measured in Wintrobe tubes centrifuged at 4,000 rpm. for 30 minutes (radius, 17 cm.). Concentration of water in plasma was estimated from plasma specific gravity (17).

All determinations were made in duplicate.

## CALCULATIONS

All plasma concentrations have been corrected for plasma water content and in the case of inulin, sucrose, and mannitol for the average increment with time in plasma blank as determined in control experiments (Figure 1). No significant changes in thiosulfate or raffinose plasma blanks were observed. Volumes of distribution of the non-electrolytes were calculated as the amount infused divided by the theoretical concentration at zero time obtained by extrapolation of the rectilinear portion of the curve of the logarithm of plasma concentration plotted against time. Assuming a Donnan factor of 1.1 for the divalent anions, sulfate and thiosulfate (3), their volumes of distribution ($V_D$) were calculated as follows:

$$V_D = Vpw + \left(\frac{Q - P \times Vpw}{P \times F}\right)$$

where Vpw equals T-1824 volume × fraction of water in plasma, Q equals quantity infused, P equals concentration of the substance in plasma water, and F equals the Donnan factor.

The virtual chloride volume was calculated by the equation above where F equals 1.05 and Q equals total exchangeable chloride minus total chloride in erythrocytes. Total exchangeable chloride was calculated from total activity infused and mean specific activity of plasma samples obtained between two and one-half to four hours after infusion. Total chloride in erythrocytes was calculated from plasma and whole blood chloride concentration, plasma volume and hematocrit.

The extrarenal clearance of each substance from its volume of distribution was calculated by the equation of Newman, Bordley, and Winternitz (15) as the product of the volume of distribution times the slope of the rectilinear portion of the plot of logarithm of concentration in plasma water against time. It is expressed as cc. per hour per Kg. body weight.

## RESULTS

Volumes of distribution of mannitol, thiosulfate and radiosulfate, simultaneously compared with either inulin, raffinose, or sucrose, are shown in Table I.

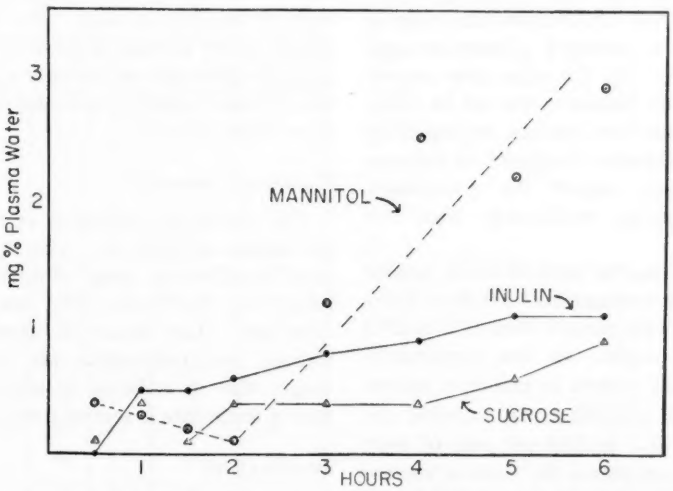


FIG. 1. INCREMENTS IN PLASMA BLANKS (AVERAGE OF THREE CONTROL EXPERIMENTS)

TABLE I

*Comparisons of volumes of distribution*

| Exp. No. | Body Wgt. | Volume of Distribution | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % of Body Weight | | | | | | % of Mannitol Volume | | | | |
| | kg. | Inulin | Raffinose | Sucrose | Radiosulfate | Thiosulfate | Mannitol | Inulin | Raffinose | Sucrose | Radiosulfate | Thiosulfate |
| 5 | 20.0 | 15.0 | | | 21.6 | 20.9 | 21.4 | 70 | | | 101 | 98 |
| 6* | 26.5 | 13.4 | | | 18.1 | + | 19.2 | 70 | | | 94 | + |
| 7 | 22.9 | 17.1 | | | 20.4 | 21.7 | 21.1 | 81 | | | 97 | 103 |
| 21 | 16.6 | 18.5 | | | not done | 22.9 | 24.3 | 76 | | | not done | 94 |
| 11 | 20.6 | | 20.2 | | 23.9 | + | 23.2 | | 87 | | 103 | + |
| 12 | 24.2 | | 21.0 | | 24.0 | 23.9 | 23.8 | | 89 | | 101 | 100 |
| 13* | 25.9 | | 16.6 | | 18.5 | + | 18.7 | | 89 | | 99 | + |
| 20 | 22.5 | | 19.8 | | 24.2 | 23.5 | 23.8 | | 83 | | 102 | 99 |
| 18 | 18.2 | | | 20.2 | 24.8 | 23.4 | 23.9 | | | 84 | 104 | 98 |
| 28 | 11.7 | | | 25.0 | 26.6 | 25.0 | 26.6 | | | 94 | 100 | 94 |
| 29 | 22.1 | | | 19.4 | 20.4 | 20.4 | 19.6 | | | 99 | 104 | 104 |
| 30 | 22.6 | | | 18.8 | 20.5 | 20.3 | 20.5 | | | 92 | 100 | 99 |
| 31 | 18.3 | | | 23.3 | 27.2 | + | 27.2 | | | 86 | 100 | + |
| | | | | | Average | | 22.6 | 74 | 87 | 91 | 100 | 99 |

* Relatively fat dogs.
\+ Fall in conc. not a simple exponential function of time.

In 13 dogs, 11 of which were lean and 2 of which relatively fat, the mannitol volume averaged 22.6 per cent of body weight (18.7 to 27.2 per cent). In 12 comparisons the ratio of radiosulfate volume to mannitol volume averaged 1.00 (0.94 to 1.03). In 9 comparisons, the ratio of thiosulfate volume to mannitol volume averaged 0.99 (0.94 to 1.05). In the other four experiments the thiosulfate volume could not be calculated because, for unknown reasons, the logarithm of thiosulfate concentration continued to decrease curvilinearly, convex toward the coordinates, rather than decreasing rectilinearly after two hours.

In four experiments the ratio of inulin volume to mannitol volume averaged 0.74 (0.70 to 0.81) as the mannitol volume ranged from 19.2 to 24.3 per cent of body weight. In four experiments the ratio of raffinose volume to mannitol volume averaged 0.87 (0.83 to 0.89) as the mannitol volume ranged from 18.7 to 23.8 per cent of body weight. In five experiments the ratio of sucrose volume to mannitol volume averaged 0.91 (0.84 to 0.99) as the mannitol volume ranged from 19.6 to 27.2 per cent of body weight. These comparisons, expressed as per cent of the simultaneously measured mannitol volume, are shown graphically in Figure 2. In Figures 3 to 5 are shown data from a representative experiment in each of the three groups. The concentration in plasma water of each of these six substances apparently decreases as a simple exponential function of time beginning one and one-half to three hours after infusion.

### *Extrarenal clearance*

The extrarenal clearances of these substances are shown in Table II. The clearance of mannitol is within the range observed in nephrectomized dogs by Houck (18), but averages somewhat less. The extrarenal clearances of inulin, sucrose, and radiosulfate are within the same range, that of raffinose is slightly higher, and that of thiosulfate is several times as high.

### *Insulin effect*

The effect of insulin on the distribution of these substances is shown in Figure 6. Insulin in an

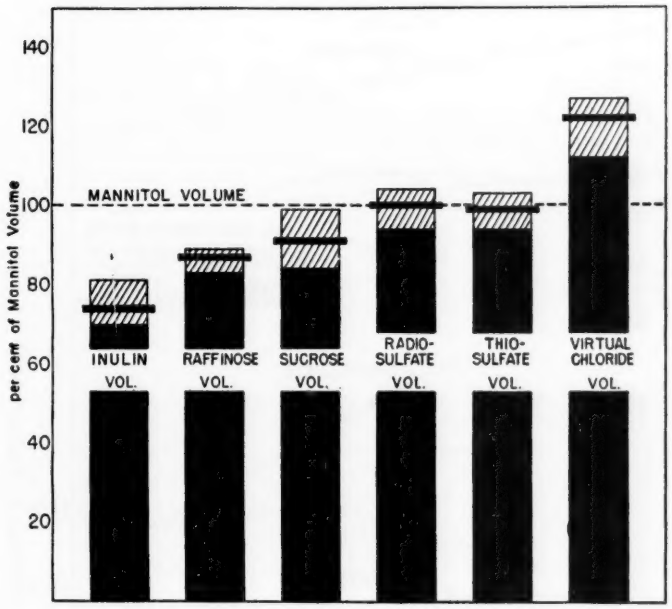


FIG. 2. COMPARISON OF VOLUMES OF DISTRIBUTION EXPRESSED AS PER CENT OF SIMULTANEOUSLY MEASURED MANNITOL VOLUME

Black horizontal line near top of each column represents average of four or five experiments. Cross-hatched area represents the range in the four or five experiments.

TABLE II

*Total extrarenal clearance*

| Exp. | CLEARANCE cc./hour/kg. | | | | | |
|---|---|---|---|---|---|---|
| | Inulin | Raffinose | Sucrose | Mannitol | Radiosulfate | Thiosulfate |
| 5 | 5.0 | | | 4.3 | 2.5 | 36 |
| 6 | 3.5 | | | 4.3 | 1.7 | |
| 7 | 3.7 | | | 4.8 | 5.8 | 29 |
| 21 | 5.3 | | | 10.9 | | 51 |
| 11 | | 5.1 | | 3.2 | 6.6 | |
| 12 | | 13.2 | | 5.5 | 2.9 | 37 |
| 13 | | 7.4 | | 3.8 | 1.3 | |
| 20 | | 9.2 | | 5.0 | 4.0 | 30 |
| 18 | | | 5.0 | 4.2 | 1.9 | 32 |
| 28 | | | 3.8 | 7.8 | 6.8 | 71 |
| 29 | | | 3.5 | 3.0 | 4.4 | 36 |
| 30 | | | 4.0 | 5.3 | 4.8 | 27 |
| 31 | | | 5.6 | 5.2 | 3.1 | |
| AV. | 4.4 | 8.7 | 4.4 | 5.4 | 3.8 | 39 |

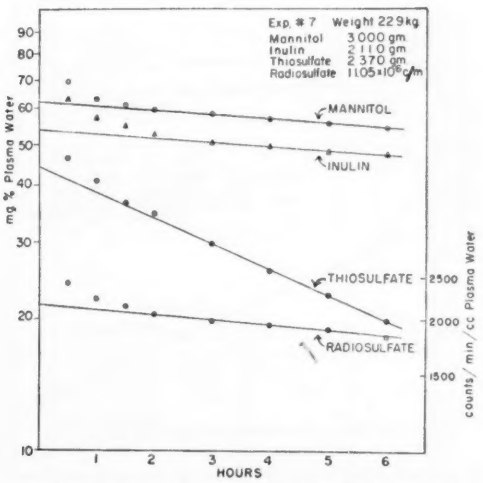


FIG. 3. CONCENTRATIONS IN PLASMA WATER OF MANNITOL, INULIN, THIOSULFATE AND RADIOSULFATE FOLLOWING INFUSION OF THESE SUBSTANCES IN AMOUNTS INDICATED INTO THE NEPHRECTOMIZED DOG AT ZERO TIME

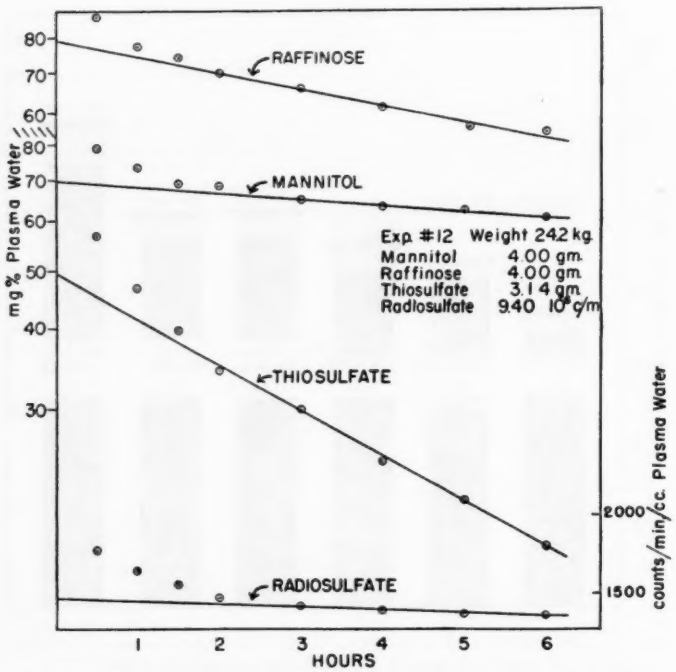


FIG. 4. CONCENTRATIONS IN PLASMA WATER OF MANNITOL, RAFFINOSE, THIOSULFATE AND RADIOSULFATE FOLLOWING INFUSION OF THESE SUBSTANCES IN AMOUNTS INDICATED INTO THE NEPHRECTOMIZED DOG AT ZERO TIME

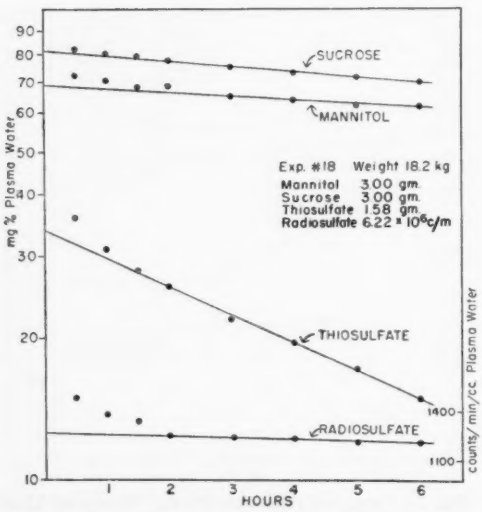


FIG. 5. CONCENTRATIONS IN PLASMA WATER OF MANNITOL, SUCROSE, THIOSULFATE, AND RADIOSULFATE FOLLOWING INFUSION OF THESE SUBSTANCES IN AMOUNTS INDICATED INTO THE NEPHRECTOMIZED DOG AT ZERO TIME

amount sufficient to depress plasma glucose concentration more than 50 per cent reduced the raffinose concentration abruptly to approximately 60 per cent of the original concentration and increased the apparent volume of distribution to 30 per cent of body weight. Insulin did not change plasma concentrations of inulin, sucrose, mannitol, and radiosulfate. The steep slope of falling thiosulfate concentration makes an evaluation of the effect of insulin less reliable. These data are omitted.

How insulin reduces raffinose concentration has not been further investigated. Conceivably, as suggested by Goldstein, Henry, Huddlestun, and Levine (8), the galactose portion of the raffinose molecule may be involved.

*Comparison of radiochloride and inulin volumes*

Data derived from the group of experiments in which inulin and radiochloride distributions were compared are shown in Table III. Total ex-

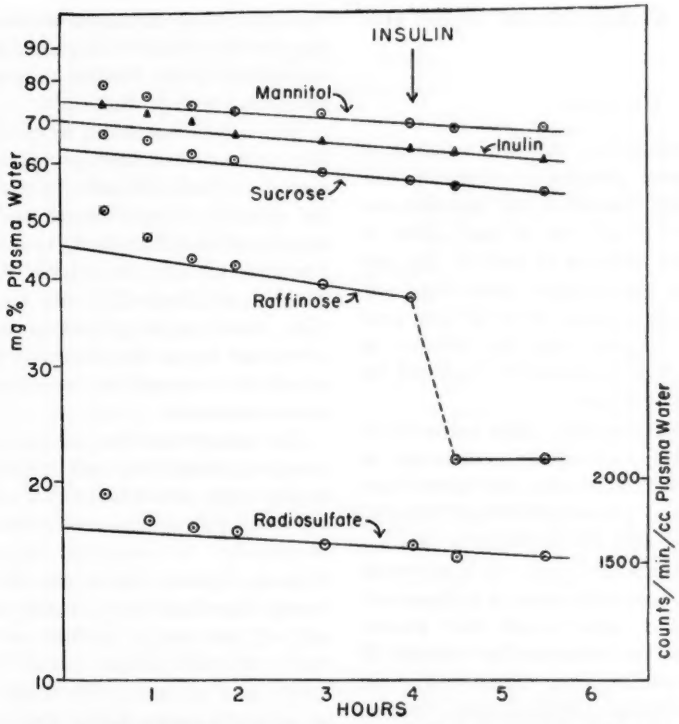


FIG. 6. EFFECT OF INTRAVENOUS INSULIN ON CONCENTRATIONS IN PLASMA WATER OF MANNITOL, INULIN, SUCROSE, RAFFINOSE AND RADIOSULFATE (COMPOSITE OF SEVERAL EXPERIMENTS)

changeable chloride averages 35.4 mM per Kg., or 637 mM in an 18 Kg. animal. When the difference between total exchangeable chloride and total erythrocyte chloride (637 minus 32 mM) is partitioned between plasma and an ultrafiltrate of plasma, the virtual chloride volume averages 27.3 per cent of body weight, while the simultaneously determined inulin volume averages 16.7 per cent of body weight. Assuming the ratio of inulin volume to mannitol volume to be 0.74, the average value obtained in the first group of experiments, the ratio of virtual chloride volume to mannitol volume can be estimated to average 1.22 under the conditions of these experiments. Of the 637 mM of total exchangeable chloride, 32 mM can be located in erythrocytes and 495 mM in the

TABLE III

*Comparison of inulin and chloride volumes of distribution*

| Exp. No. | Wgt. Kg. | Plasma Water Volume | Chloride Conc. in Plasma Water | Total Exchange. Chloride | Total RBC Chloride | Virtual Chloride Volume | | Inulin Volume | | Measured $V_{Cl^-}/V_{In.}$ | Calculated $V_{Cl^-}/V_{Mann.}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | cc. | mM./L. | mM./Kg. | mM. | cc. | % Body Wgt. | cc. | % Body Wgt. | | |
| 22A | 19.6 | 1110 | 119.5 | 34.5 | 42 | 5600 | 28.5 | 3035 | 15.5 | 1.85 | 1.37 |
| 23A | 23.6 | 1160 | 118.0 | 36.5 | 35 | 6725 | 28.5 | 4220 | 17.9 | 1.60 | 1.18 |
| 24A | 16.5 | 750 | 118.5 | 32.5 | 38 | 4040 | 24.5 | 2670 | 16.2 | 1.52 | 1.12 |
| 25A | 16.3 | 770 | 113.6 | 37.2 | 28 | 4540 | 27.9 | 2940 | 18.0 | 1.55 | 1.15 |
| 20A | 14.1 | 620 | 115.0 | 36.7 | 25 | 3860 | 27.3 | 2210 | 15.7 | 1.75 | 1.29 |

mannitol volume, leaving 110 mM located elsewhere.

## DISCUSSION

These data demonstrate that the volumes of distribution of three chemically dissimilar substances, radiosulfate, thiosulfate, and mannitol, are consistently within 6 per cent of each other as body weight varies between 12 and 27 Kg. and as the per cent of body weight which these volumes represent ranges from 19 to 27 per cent. This observation suggests that the volumes of distribution of these three substances represent the same portion of body water.

Is this volume coextensive with extracellular fluid when the latter is defined, essentially as Edelman, Olney, James, Brooks, and Moore have (19), as plasma plus plasma ultrafiltrate bounded by capillary membranes, cell membranes, and extracellular solid structures (*e.g.,* hydroxyapatite crystals of bone or macromolecules in collagen and elastic fibers)? For cerebrospinal fluid, gastrointestinal secretion, and other smaller volumes of fluid which are separated from extracellular fluid by epithelium, Edelman, Olney, James, Brooks, and Moore suggest the term "transcellular fluid." Cerebrospinal fluid is not included in extracellular fluid as here defined. Neither is it included in the calculated volumes of distribution of these substances, because under the conditions of these experiments, concentrations of radiosulfate, mannitol, sucrose, and inulin in cerebrospinal fluid obtained from a lateral ventricle or the cisterna magna six hours after infusion are less than 10 per cent of the simultaneous plasma concentrations (20). Gastrointestinal secretions are not included in extracellular fluid as here defined. Under these experimental conditions, radiosulfate, mannitol, and sucrose appear in bile and pancreatic juice in concentrations approaching plasma concentration, while inulin is excreted in bile in a concentration approximately one-third that of plasma (20).

Inulin and sucrose are hydrolyzed in the gut and presumably their calculated volumes of distribution do not include gastrointestinal secretion or contents.

If mannitol (21) and radiosulfate are reabsorbed from the gut at these concentrations, a fraction of their calculated volumes of distribution may include a fraction of gastrointestinal fluid, the magnitude of this fraction depending on the rate and site of reabsorption (22).

The volume measured by radiosulfate, thiosulfate, and mannitol includes approximately 80 per cent of the body chloride. The remainder, minus the chloride in erythrocytes and estimated chloride in transcellular fluid, is consistent with the fractions of chloride which Amberson, Nash, Mulder, and Binns (23) and Yannet and Darrow (24) found to be present in tissues in a form other than as an ultrafiltrate of plasma, either intracellular or bound in some manner to extracellular structures.

The concept that the volumes of distribution of mannitol, thiosulfate, and radiosulfate are coextensive with extracellular fluid as here defined is not compatible with available information on their distribution as measured by tissue analysis. Nichols, Nichols, Weil, and Wallace (7) have found thiosulfate to be virtually equilibrated with only 45 per cent of Achilles or patellar tendon water and with 78 per cent of skin water three hours after infusion. The bulk of tendon water is certainly extracellular, but its total volume in studies on the whole animal probably is not significant. However, even the most generous estimate of intracellular water in skin can account for only one-half to one-third of the water of skin into which thiosulfate fails to distribute. The volume of this water is probably significant in studies on the whole animal. Studies of the distribution into water of skin and bone of radiosulfate or mannitol, substances whose plasma concentrations do not decrease as rapidly as does that of thiosulfate, should answer whether significant fractions of extracellular fluid can be shown to be excluded from these volumes of distribution.

A truly rectilinear decrease in the logarithm of the plasma concentration of radiosulfate, thiosulfate, mannitol, sucrose, raffinose, and inulin, with time after one and one-half to three hours of equilibration, would indicate that these substances are being removed from their calculated volumes of distribution by such processes as destruction within cells or destruction or sequestration in the gut. It would exclude diffusion into more restricted or remote regions of extracellular fluid at relatively slower rates. The latter possibility

is suggested by the studies of Cotlove (25) and Nichols, Nichols, Weil, and Wallace (7) on the distribution of inulin, sucrose, and thiosulfate in tissues. Our data do not exclude this possibility because the very slight curvilinearity in the plot reflecting such a process would not become apparent during the period of these observations.

Despite the above uncertainties regarding the identification of the volumes of distribution of radiosulfate, thiosulfate, and mannitol with extracellular fluid, we believe their distributions are the best available measure of extracellular fluid volume in the nephrectomized dog. Disadvantages are: variability in the plasma blank in the case of mannitol, the occasional failure to observe a linear decrease in the logarithm of plasma concentration with time in the case of thiosulfate, and the uncertainty regarding Donnan distribution across capillary membranes in the case of radiosulfate and thiosulfate.

We are unable to rationalize on a structural basis the smaller volumes of distribution of sucrose, raffinose, and inulin. In the nephrectomized dog these volumes are clearly smaller than those of radiosulfate, thiosulfate, and mannitol. The question whether similar relations obtain in man could be answered by similar comparisons in anuric patients.

## SUMMARY

1. The volumes of distribution of radiosulfate, thiosulfate, mannitol, sucrose, raffinose, and inulin have been compared in nephrectomized dogs. Radiosulfate, thiosulfate, and mannitol volumes are apparently equal when measured simultaneously in the same animal. The volumes of distribution of sucrose, raffinose, and inulin are smaller in inverse order to the molecular size of these substances.

2. Intravenous insulin, which has been shown by others to affect the distribution of other slowly metabolized monosaccharides, increases the apparent volume of distribution of raffinose, but does not affect the distribution of radiosulfate, mannitol, sucrose, or inulin.

3. The possibility that the volume measured by radiosulfate, thiosulfate, and mannitol distribution is coextensive with extracellular fluid, as here defined, is discussed.

## ACKNOWLEDGMENT

We are indebted to our associate, Dr. Henry D. Lauson, for his criticism of the manuscript.

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# URINARY PROTEIN EXCRETION AND RENAL HEMODYNAMIC ADJUSTMENTS DURING ORTHOSTASIS IN PATIENTS WITH ACUTE AND CHRONIC RENAL DISEASES [1, 2]

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(Submitted for publication March 5, 1954; accepted July 7, 1954)

The amount and character of the protein lost in the urine may vary widely during the course of renal disease (2). Since proteinuria seems to result from increased permeability of the glomerular capillaries (2, 3) (though a reduction in tubular reabsorption of protein may also play a role [4]), variation in proteinuria may be ascribed chiefly to changes in glomerular function. Progression or healing of the renal lesion undoubtedly alters the structure and area of the glomerular filtering surface with resultant change in the escape of protein from the plasma into the urine (5–8). In addition, proteinuria is affected by renal circulatory adjustments that may influence filtration. The renal vascular responses to congestive heart failure, shock, fever, violent exercise or pressor drugs such as epinephrine, are often associated with the appearance of proteinuria in normal persons and with augmentation of proteinuria in patients with renal disease (5, 9–12). Less striking changes in the renal circulation, such as those associated with assumption of the upright position, do not usually result in loss of protein by the normal kidney. Pre-existing proteinuria, on the other hand, may increase during orthostasis, suggesting that glomerular permeability may be more susceptible to change in the diseased kidney. The effect of orthostasis upon glomerular filtration, renal blood flow, and protein excretion was studied in patients with acute or chronic renal diseases in order to define more accurately the role of renal hemodynamic alterations in producing variation in proteinuria.

## METHODS

Twenty studies were made in 19 fasting patients with well-documented renal disease: Acute diffuse glomerulonephritis (four), chronic diffuse glomerulonephritis (six), benign nephrosclerosis (three), chronic pyelonephritis (two), disseminated lupus erythematosus (two), malignant nephrosclerosis (one), and multiple myeloma without Bence-Jones proteinuria (one). Two subjects (W. M. and M. M.) with chronic diffuse glomerulonephritis also suffered from the nephrotic syndrome. Two studies were performed on one subject (M. M.).

An effort was made to select subjects with marked proteinuria as judged by the heat-acetic acid test. However, since this method provides only a rough estimate of the concentration of urinary protein, some subjects excreting less than four grams of protein per day were included in the series.

The glomerular filtration rate was measured as the inulin clearance, and effective renal plasma flow as the p-aminohippurate [5] clearance (9, 13). Tubular function was assessed in terms of the maximal rate of glucose reabsorption by the tubules (glucose $T_m$) (9, 13). Urinary protein concentration was determined by the micro-Kjeldahl method (7) and plasma protein concentration in blood collected without stasis by a specific gravity (copper sulfate) method (14). Total protein clearance was calculated from the total excretion (mg. per min.) divided by the total plasma concentration (mg. per ml.). This calculation is useful in expressing alterations in total protein output relative to the plasma concentration provided changes in individual protein fractions of plasma or urine do not occur. Blood urea nitrogen was measured (15) in all but one subject (J. W.). The blood pressure was measured sphygmomanometrically and the mean pressure calculated as the diastolic pressure plus 40 per cent of the pulse pressure (16).

In seven subjects urinary and serum or plasma protein patterns were determined by paper electrophoresis (17)

[1] This work was supported by a grant from the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under contract No. DA-49-007-MD-205.

[2] These data have been presented in preliminary form elsewhere (1).

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[5] Supplied through the courtesy of Dr. William Boger, Sharpe and Dohme, Inc., Glenolden, Penna.

before and during orthostasis and on return to the horizontal. In an attempt to obtain sufficient protein concentrations for clear resolution and identification, aliquots of pooled urine (usually 5 to 15 ml.) collected during the control periods and aliquots of single urine samples collected during the upright and recovery periods were reduced to volumes of less than 1 ml., by dialysis overnight through a cellophane bag against 20 per cent Dextran[6] or 25 per cent polyvinyl pyrollidone.[7] Denaturation or destruction of proteins did not occur provided the contents of the bag were not reduced to dryness.

Following determination of control clearance values during two or three successive ten to twenty-minute intervals with the subject in the recumbent position, clearance values were determined during intervals of ten to twenty-five minutes in the passive upright position with the subject tilted on a tilt table to 40 to 80° (11 subjects) or half-sitting, half-standing at the end of a bed (8 subjects). Since urine flow fell abruptly and since weakness and hypotension frequently developed within ten to fifteen minutes after standing, only one clearance period could be obtained. Urine collected during the first ten to fifteen minutes of orthostasis was discarded in all but five instances (S. G., H. T., H. Y., M. S., M. L.) in order to permit equilibration. The total interval during the upright position, therefore, varied from twenty to thirty-five minutes. On twelve occasions clearance values were determined over an interval of from fifteen to thirty-five minutes (one or two periods) immediately after the return to the horizontal (recovery). In order to determine whether reduction in clearance values during orthostasis could be attributed to a failure to achieve equilibrium owing to delay in the passage of urine from the glomerulus to the bladder, the urine collected during the first period immediately after return to the horizontal was not discarded. Inclusion of the values for this period in the average values for "recovery" would produce higher values than in the control ("overshoot") if intrarenal delay were an important factor. Glucose $T_m$ was determined in six subjects before and during orthostasis in order to assess the distribution of filtrate to functioning tubular tissue. Urine collected during the first ten to fifteen minutes of orthostasis was discarded. Values during recovery (without discard) were measured in five subjects.

## RESULTS

### *Systemic circulatory adjustments and subjective response*

Within five to thirty minutes after assumption of the upright position evidence of mild to severe circulatory insufficiency (dizziness, sweating, pallor, nausea) appeared in the course of ten of twenty studies (E. A., S. G., H. O'N., J. McC., B. W., M. M. No. 1, M. M. No. 2, A. C., E. B., and R. W.). In six of seven subjects in whom arterial pressures were measured (Table I) both systolic and diastolic pressures tended to fall. Although the seventh subject (R. W.), became quite pale and dizzy his arterial pressure was maintained. In all but S. G., who was able to remain upright despite slight weakness and nausea, immediate return to recumbency was necessary in order to prevent syncope. The arterial pressure then returned promptly toward or to control levels and symptoms of circulatory insufficiency disappeared in all but E. B. and R. W., in whom weakness and faintness persisted during the first fifteen minutes of recovery. In the remaining subjects circulatory adjustments during orthostasis were apparently adequate and symptoms of circulatory insufficiency did not develop.

### *Inulin and sodium p-aminohippurate (PAH) clearances*

The averaged figures for inulin and PAH clearances during the control period, during orthostasis and during recovery are arranged in Table I ($C_{in}$ and $C_{PAH}$) according to the control values for the inulin clearances, in descending order. The responses to tilting upright and to standing at the edge of the bed were qualitatively similar, although somewhat greater percentile changes occurred in subjects who were passively tilted. During orthostasis the PAH clearance fell definitely (on the average 41 per cent) in 17 of 20 studies and the inulin clearance decreased similarly (on the average 41 per cent) in 17 instances (Table I, Figure 1). The PAH clearance was unchanged in three subjects (S. G., R. W., and A. D.) and the inulin clearance in three (S. G., B. W., and A. D.). The inulin and PAH clearances tended to fall proportionately so that the filtration fraction (F. F., Table I) was not greatly changed. The excessively high values for the filtration fractions in S. G., J. McC., E. B., M. S., and R. W. are probably the result of incomplete extraction of PAH by the diseased kidneys.

On return to the horizontal position the inulin and PAH clearances promptly returned to or toward control levels in all but two (E. B., and R. W., Table I) of twelve studies. In E. B. and R. W., the averaged values for the two recovery

[6] Provided through the courtesy of Commercial Solvents Corp., Terre Haute, Indiana.

[7] Provided through the courtesy of Schenley Laboratories, Inc., Lawrenceburg, Indiana.

periods and in S. G. the values during the single "recovery" period (Table I) did not return to control levels owing to continued depression of function and/or intra-renal delay during the first fifteen minutes of recumbency. However, during the last recovery period the clearance values returned to the control level in both E. B. and R. W. In one subject (H. T.) the clearance values during recovery were somewhat higher than control figures, but in other subjects the control values were not exceeded.

The absence of an "overshoot" in clearance values, as urine flow increased, during recovery strongly supports the view that delay of filtrate

TABLE I†

*Renal function and protein excretion during orthostasis* *

| Sex | Subject Diagnosis Age | S.A. | BUN | Procedure | B.P. | T.P. | Hct. | $C_{in}$ | $C_{PAH}$ | FF | V | $U_{Pr}$ | $U_{Pr}V$ | $U_{Pr}V/C_{in}$ | $C_{Pr}$ | $C_{Pr}/C_{in} \times 10^{-3}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $M^2$ | mg. % | | mm. Hg | gm. % | % | ml./min. | ml./min. | % | ml./min. | mg. % | mg./min. | μg./ml. | ml./min. | % |
| M | E. A. Acute glom. neph. 48 | 2.02 | 19 | Control | 124/84 | 7.03 | 41 | 131 | 488 | 27 | 1.7 | 179 | 3.1 | 23 | 0.05 | 0.3 |
| | | | | Orthostasis | — | 7.03 | 41 | 23 | 79 | 29 | 0.2 | 320 | 0.6 | 28 | 0.01 | 0.4 |
| M | S. G. Chronic pyeloneph. 25 | | 14 | Control | 120/80 | 6.35 | 51 | 123 | 196 | 63 | 2.8 | 268 | 7.5 | 61 | 0.12 | 1.0 |
| | | | | Orthostasis | 106/62 | 6.72 | 53 | 114 | 191 | 60 | 1.9 | 211 | 4.0 | 35 | 0.06 | 0.5 |
| | | | | Recovery | — | 6.72 | 50 | 110 | 120 | 58 | 1.3 | 462 | 6.0 | 55 | 0.09 | 0.8 |
| M | N. H. Mult. myeloma 51 | 1.66 | 16 | Control | 120/80 | 6.72 | 41 | 118 | 660 | 18 | 1.0 | 600 | 6.0 | 51 | 0.09 | 0.8 |
| | | | | Orthostasis | — | 6.72 | 41 | 79 | 411 | 19 | 1.4 | 336 | 4.7 | 59 | 0.07 | 0.9 |
| F | L. DiA. Diss. lupus erythem. 28 | 1.56 | 7 | Control | 115/70 | 5.97 | 34 | 105 | 708 | 15 | 3.9 | 108 | 4.2 | 40 | 0.07 | 0.7 |
| | | | | Orthostasis | — | 5.97 | 36 | 34 | 160 | 21 | 0.8 | 275 | 2.2 | 65 | 0.04 | 1.2 |
| | | | | Recovery | — | 5.97 | — | 110 | 505 | 22 | 0.6 | 667 | 4.0 | 36 | 0.07 | 0.6 |
| F | J. W. Benign nephroscler. 33 | 1.70 | — | Control | 160/120 | 6.00 | 39 | 105 | 484 | 22 | 8.5 | 12 | 1.0 | 10 | 0.02 | 0.2 |
| | | | | Orthostasis | — | 6.00 | — | 63 | 266 | 24 | 0.9 | 112 | 1.0 | 16 | 0.02 | 0.3 |
| M | H. O'N. Acute glom. neph. 28 | 1.91 | 17 | Control | 140/90 | 5.97 | 49 | 101 | 511 | 20 | 2.3 | 209 | 4.8 | 48 | 0.08 | 0.8 |
| | | | | Orthostasis | — | 6.34 | 49 | 66 | 291 | 23 | 0.3 | 0 | 0.0 | 0 | 0.00 | 0.0 |
| M | H. T. Acute glom. neph. 26 | 1.69 | 20 | Control | 116/110 | 5.97 | 49 | 92 | 604 | 15 | 4.9 | 110 | 5.4 | 59 | 0.09 | 1.0 |
| | | | | Orthostasis | — | 5.97 | 49 | 38 | 223 | 17 | 1.7 | 194 | 3.3 | 87 | 0.06 | 1.6 |
| | | | | Recovery | — | 5.97 | — | 108 | 761 | 14 | 1.8 | 239 | 4.3 | 40 | 0.07 | 0.7 |
| F | M. C. Diss. lupus erythem. 20 | 1.56 | 23 | Control | 110/70 | 5.41 | 32 | 63 | 482 | 13 | 5.5 | 40 | 2.2 | 36 | 0.04 | 0.7 |
| | | | | Orthostasis | — | 5.41 | 31 | 33 | 267 | 12 | 3.2 | 25 | 0.8 | 23 | 0.01 | 0.4 |

* All control clearance values are averages of two or more determinations. All other clearance values represent single determinations except in instances marked with an asterisk, which are averages of two determinations.

† Abbreviations are as follows:

S.A. = surface area ($M^2$)
BUN = blood urea nitrogen (mg./100 ml.)
B.P. = arterial pressure determined sphygmomanometrically (mm. Hg)
T.P. = total serum protein (gm./100 ml.)
Hct. = red cell hematocrit (%)
$C_{in}$ = inulin clearance (ml./min.)
$C_{PAH}$ = sodium p-aminohippurate clearance (ml./min.)
FF = filtration fraction ($C_{in}/C_{PAH}$)
V = urine flow (ml./min.)
$U_{Pr}$ = urinary protein concentration (mg./100 ml.)
$U_{Pr}V$ = urinary protein output (mg./min.)
$U_{Pr}V/C_{in}$ = urinary protein output relative to filtration, or, protein concentration per unit of filtrate (μg./ml.)
$C_{Pr}$ = protein clearance (ml./min.)
$C_{Pr}/C_{in}$ = protein-inulin clearance ratio

TABLE I—*Continued*

| Sex | Subject Diagnosis Age | S.A. | BUN | Procedure | B.P. | T.P. | Hct. | $C_{In}$ | $C_{PAH}$ | FF | V | $U_{Pr}$ | $U_{Pr}V$ | $U_{Pr}V/C_{In}$ | $C_{Pr}$ | $C_{Pr}/C_{In} \times 10^{-3}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *M²* | *mg. %* | | *mm. Hg* | *gm. %* | *%* | *ml./min.* | *ml./min.* | *%* | *ml./min.* | *mg. %* | *mg./min.* | *µg./ml.* | *ml./min.* | *%* |
| M | J. McC. Benign nephroscler. 54 | 1.74 | 24 | Control | 250/150 | 6.34 | 44 | 59 | 126 | 47 | 4.2 | 67 | 2.8 | 47 | 0.04 | 0.7 |
| | | | | Orthostasis | 200/140 | 6.72 | 46 | 37 | 98 | 38 | 0.8 | 200 | 1.6 | 43 | 1.02 | 0.6 |
| M | W. M. Chronic glom. neph. 44 | 1.86 | 22 | Control | 160/100 | 4.01 | 30 | 57 | 467 | 12 | 5.0 | 140 | 7.0 | 120 | 0.18 | 3.2 |
| | | | | Orthostasis | 138/96 | 4.48 | 33 | 40 | 312 | 13 | 1.9 | 363 | 6.9 | 170 | 0.15 | 3.8 |
| | | | | Recovery* | 132/70 | 4.48 | 32 | 54 | 470 | 11 | 2.5 | 196 | 4.9 | 90 | 0.11 | 2.0 |
| F | B. W. Acute glom. neph. 15 | 1.83 | 14 | Control | 106/66 | 6.72 | 40 | 55 | 432 | 13 | 4.7 | 53 | 2.5 | 50 | 0.04 | 0.7 |
| | | | | Orthostasis | 70/30 | 7.09 | — | 48 | 313 | 15 | 2.4 | 53 | 1.6 | 33 | 0.02 | 0.4 |
| | | | | Recovery | 110/70 | 6.90 | 39 | 54 | 360 | 15 | 2.4 | 63 | 1.5 | 30 | 0.02 | 0.4 |
| M | H. Y. Benign nephroscler. 26 | 1.78 | 15 | Control | 180/100 | 5.22 | 48 | 48 | 169 | 28 | 1.9 | 479 | 9.1 | 190 | 0.17 | 3.5 |
| | | | | Orthostasis | — | 5.60 | 50 | 37 | 114 | 32 | 0.7 | 972 | 6.8 | 180 | 0.13 | 3.5 |
| | | | | Recovery | — | 4.85 | 48 | 54 | 170 | 32 | 0.9 | 1266 | 11.4 | 210 | 0.23 | 4.3 |
| F | M. M. No. 1 Chronic glom. neph. 22 | 1.60 | 31 | Control | 194/100 | 4.85 | 37 | 31 | 193 | 16 | 1.9 | 484 | 9.2 | 300 | 0.19 | 6.1 |
| | | | | Orthostasis | 120/80 | 4.85 | 37 | 12 | 74 | 16 | 0.4 | 626 | 2.5 | 210 | 0.05 | 4.3 |
| | | | | Recovery | 192/120 | 4.66 | 35 | 19 | 126 | 15 | 0.6 | 850 | 5.1 | 270 | 0.11 | 5.7 |
| M | A. C. Chronic glom. neph. 37 | 1.78 | 41 | Control | 200/120 | 8.21 | 37 | 26 | 102 | 26 | 6.9 | 144 | 9.9 | 370 | 0.12 | 4.0 |
| | | | | Orthostasis | 180/120 | 8.58 | 38 | 17 | 64 | 27 | 2.8 | 61 | 1.7 | 120 | 0.02 | 1.0 |
| | | | | Recovery | 198/120 | 7.84 | 38 | 26 | 99 | 27 | 4.6 | 143 | 6.8 | 260 | 0.09 | 3.0 |
| F | M. M. No. 2 Chronic glom. neph. 22 | 1.60 | 31 | Control | 160/100 | 4.85 | 36 | 22 | 185 | 12 | 2.6 | 238 | 6.2 | 280 | 0.13 | 5.9 |
| | | | | Orthostasis | 90/50 | 4.85 | 36 | 2 | 15 | 12 | 0.1 | 60 | 0.6 | 300 | 0.01 | 5.0 |
| | | | | Recovery* | 140/90 | 4.66 | 35 | 20 | 188 | 11 | 0.8 | 650 | 5.2 | 260 | 0.11 | 5.1 |
| M | E. B. Malig. nephroscler. 56 | 2.01 | 39 | Control | 200/110 | 6.72 | 30 | 20 | 28 | 71 | 3.3 | 94 | 3.1 | 160 | 0.08 | 2.4 |
| | | | | Orthostasis | — | — | — | 14 | 21 | 67 | 1.5 | 133 | 2.0 | 140 | 0.03 | 2.1 |
| | | | | Recovery* | — | 6.72 | 29 | 14 | 24 | 58 | 1.5 | 86 | 1.3 | 90 | 0.02 | 1.5 |
| M | M. S. Chronic pyeloneph. 26 | 2.06 | 41 | Control | 210/130 | 5.97 | 27 | 9 | 19 | 47 | 4.1 | 200 | 8.2 | 910 | 0.14 | 16.0 |
| | | | | Orthostasis | — | 6.34 | 28 | 4 | 10 | 40 | 1.6 | 256 | 4.1 | 1020 | 0.06 | 15.0 |
| M | R. W. Chronic glom. neph. 58 | 1.70 | 93 | Control | 180/100 | 7.84 | 20 | 8 | 13 | 62 | 3.7 | 108 | 4.0 | 490 | 0.05 | 6.0 |
| | | | | Orthostasis | 180/92 | 7.84 | 20 | 6 | 12 | 50 | 2.7 | 74 | 2.0 | 340 | 0.03 | 4.0 |
| | | | | Recovery* | 190/90 | 7.84 | 20 | 6 | 11 | 54 | 2.4 | 105 | 2.5 | 420 | 0.03 | 4.0 |
| F | M. L. Chronic glom. neph. 33 | 1.68 | 105 | Control | 210/110 | 5.22 | 19 | 5 | 69 | 8 | 1.8 | 183 | 3.3 | 630 | 0.06 | 12.0 |
| | | | | Orthostasis | — | 5.97 | 19 | 2 | 40 | 6 | 0.8 | 225 | 1.8 | 780 | 0.03 | 13.0 |
| | | | | Recovery | — | | | 3 | 62 | 5 | 1.1 | 73 | 0.8 | 260 | 0.01 | 3.0 |
| M | A. D. Chronic glom. neph. 35 | 1.49 | 126 | Control | 110/80 | 6.71 | 27 | 4 | 30 | 14 | 1.2 | 217 | 2.6 | 60 | 0.04 | 9.4 |
| | | | | Orthostasis | — | 6.34 | 25 | 4 | 30 | 14 | 1.1 | 64 | 0.7 | 17 | 0.01 | 2.4 |

within the tubules during orthostasis did not contribute importantly to the changes observed during orthostasis. These changes may be ascribed to decrements in renal blood flow and glomerular filtration. Since impaired renal extraction of PAH is characteristic of chronic renal disease (9), a further acute reduction in extraction might account for the fall in PAH clearance. However, the degree of fall in PAH clearance, its association with a similar decrease in inulin clearance and its reversibility support the view that diminished PAH excretion under these circumstances was the result of reduced delivery of PAH by the blood to tubular cells.

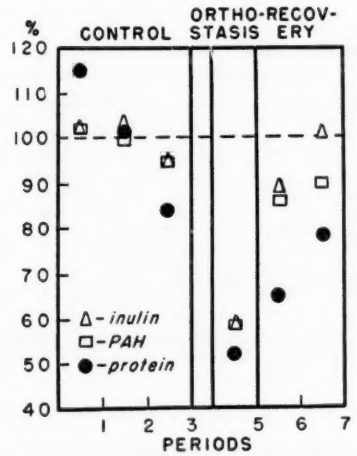


FIG. 1. THE EFFECT OF ORTHOSTASIS ON THE CLEARANCES OF INULIN AND PAH AND ON URINARY PROTEIN EXCRETION

The values for inulin and PAH clearances and for protein output have been averaged for each period and expressed as the percentage of the pooled and averaged control figures. All data presented here represent complete experiments in twelve subjects (Table I) except for the second recovery period which represent values of four subjects (W. M., M. M. No. 2, E. B., and R. W.) only. All values fell sharply and proportionately during orthostasis and returned toward control levels during recovery.

*Tubular transfer maximum (glucose $T_m$)*

The maximal rate of tubular reabsorption of glucose was measured in six subjects (Table II). In all inulin clearance and glucose $T_m$ fell to approximately the same extent, so that the inulin clearance-glucose $T_m$ ratio ($C_{in}/T_{mg}$, Figure 2) remained relatively constant. During recovery glucose $T_m$ returned to control levels in four of the five instances studied.

Whether complete saturation of all tubules had been effected in these subjects could not be determined with certainty. Even at high plasma glucose levels saturation of the tubules may be difficult to achieve in advanced renal disease because filtration may be so greatly reduced in many nephrons. When the load imposed upon the tubules is reduced by a further fall in filtration, such as that which occurs during orthostasis, glucose $T_m$ may appear to decrease simply because the load has decreased in proportion to filtration. The filtered load of glucose fell below the control value for the maximal rate of reabsorption in J. McC. during orthostasis (load/$T_{mg}$ [Table II] ratio less than one). In W. M. the load/$T_{mg}$ ratio fell to 1.04, but in E. B., B. W., and A. C. the load remained much higher than the control value for $T_{mg}$. In view of the fall in $T_{mg}$ even when the load was sustained at a high level and the proportionate reduction in $T_{mg}$ and inulin clearance even when the calculated load decreased below the control rate of glucose reabsorption it seems most likely that the decrement in $T_{mg}$ may be ascribed to cessation of filtration in a proportion of the glomeruli. Since an excess of glucose was not excreted during recovery, it may be inferred that delay within the urinary passages did not contribute significantly to the fall in glucose $T_m$ during orthostasis. The changes noted in $C_{in}/T_{mg}$ ratio during recovery cannot be explained but they are possibly attributable to inclusion of urine collected during the period of equilibration immediately after return to the horizontal in order to detect "overshoot."

*Protein excretion*

The excretion of protein in the urine decreased sharply in 18 instances, and remained unchanged in two (J. W. and W. M.) ($U_{pr}V$, Table I). The failure to detect a change in proteinuria in J. W. may have been due to technical errors since the control urinary protein concentration (12 mg. per

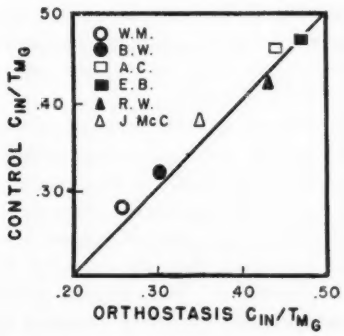


FIG. 2. DISTRIBUTION OF GLOMERULAR FILTRATE TO FUNCTIONING TUBULAR TISSUE

The filtration rate ($C_{in}$) relative to maximal glucose reabsorptive capacity ($T_{mg}$) during the control period is plotted against this ratio during orthostasis. In all subjects filtration and $T_{mg}$ fell proportionately, presumably indicating that filtration diminished entirely as a result of cessation of glomerular activity.

TABLE II

*Effect of orthostasis upon maximal glucose reabsorptive capacity ($T_{mg}$)* *

| Subject | Control | | | | Orthostasis | | | | Recovery | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plasma glucose | Load† / $T_{mg}$ | Glucose $T_m$ | $C_{in}/T_{mg}$‡ | Plasma glucose | Load† / $T_{mg}$ | Glucose $T_m$ | $C_{in}/T_{mg}$‡ | Plasma glucose | Load† / $T_{mg}$ | Glucose $T_m$ | $C_{in}/T_{mg}$‡ |
| | *mg. %* | | *mg./min.* | | *mg. %* | | *mg./min.* | | *mg. %* | | *mg./min.* | |
| J. McC. | 400 | 1.5 | 158 | 0.38 | 335 | 0.8 | 102 | 0.36 | — | — | — | |
| W. M. | 487 | 1.3 | 206 | 0.28 | 525 | 1.04 | 151 | 0.27 | 534 | 1.4 | 206* | 0.26 |
| B. W. | 632 | 2.0 | 173 | 0.32 | 574 | 1.6 | 156 | 0.31 | 560 | 1.7 | 188 | 0.29 |
| A. C. | 601 | 2.8 | 59 | 0.46 | 614 | 1.8 | 39 | 0.44 | 620 | 2.8 | 65 | 0.40 |
| E. B. | 378 | 1.8 | 43 | 0.47 | 395 | 1.3 | 30 | 0.47 | 417 | 1.4 | 38* | 0.37 |
| R. W. | 609 | 2.6 | 19 | 0.42 | 650 | 2.1 | 14 | 0.43 | 698 | 2.2 | 11* | 0.55 |

* All control values are averages of three determinations. All other values represent single determinations except those instances marked with an asterisk (*), which are averages of two periods. Values for urine flow, $C_{in}$ and $C_{PAH}$ obtained simultaneously in these patients are presented in Table I.

Abbreviations as in Table I except as follows:

† Load/$T_{mg}$ = glucose filtered (arterial glucose concentration (mg./ml.) × inulin clearance (ml./min.)) relative to maximal reabsorptive capacity ($T_{mg}$).

‡ $C_{in}/T_{mg}$ = glomerular filtration rate relative to maximal tubular reabsorptive capacity (ml./mg.), using the value for $T_{mg}$ obtained during the control periods.

cent) was too low for accurate measurement. The excretion of protein varied widely from subject to subject (1.0 to 9.9 mg. per min.) and showed a tendency to fall slightly during the control periods. Nevertheless, the fall in protein excretion during orthostasis is highly significant ($t = 2.81$, $p < .01$) when compared with spontaneous variations in proteinuria observed over a similar time interval in a group of eight proteinuric subjects studied during recumbency only.

On returning to the horizontal position (12 studies) protein output tended to return toward, or to, control levels in all but five subjects (E. B., R. W., W. M., B. W., and M. L.) in whom the reduction in protein excretion persisted. The control value was exceeded on only one occasion (H. Y.) during recovery.

On the average, the excretion of urinary protein decreased 49 per cent during orthostasis, a value corresponding closely to the average changes in inulin and PAH clearances (Figure 1). The return of protein excretion to control levels was somewhat less rapid than the recovery in inulin and PAH clearances. Since plasma protein concentration remained relatively constant in most subjects total protein clearances showed directional and percentile changes similar to those for total protein excretion (Table I). However, in a few subjects the plasma protein concentrations increased slightly owing to hemoconcentration (parallel increments in the hematocrit were usually observed) during gravitational pooling of blood in the lower extremities; hence, protein clearances tended to change less than protein output. Protein excretion fell relatively more than urine flow in eight instances (S. G., N. H., H. O'N., M. C., A. C., M. M. No. 2, R. W., and A. D.), and urinary protein concentration decreased. In B. W. the urinary concentration of protein did not change but in the remaining eleven studies it increased.

In general the decrease in protein excretion during orthostasis was roughly proportional to the fall in filtration, but disproportionate reductions in proteinuria did occur. Protein excretion and clearance fell less in five subjects (L. D'A., S. W., H. T., W. M., and M. L.) and more in eight (S. G., H. O'N., M. C., B. W., M. M. No. 1, A. C., R. W., and A. D.) than the inulin clearance (Table I). In J. W. and W. M. the inulin clearance fell 40 and 30 per cent, respectively, without changes in protein output, whereas in A. D. protein excretion fell significantly without an apparent change in filtration. However, in A. D. the inulin clearance was so greatly reduced by disease (4 ml. per min.) that a further significant reduction may have been too small to detect.

Satisfactory patterns of plasma (or serum) and urinary proteins by paper electrophoresis were obtained in seven subjects before and during orthostasis. Owing to difficulty in photographic reproduction (lack of contrast) the results of four experiments only are illustrated in Figure 3.

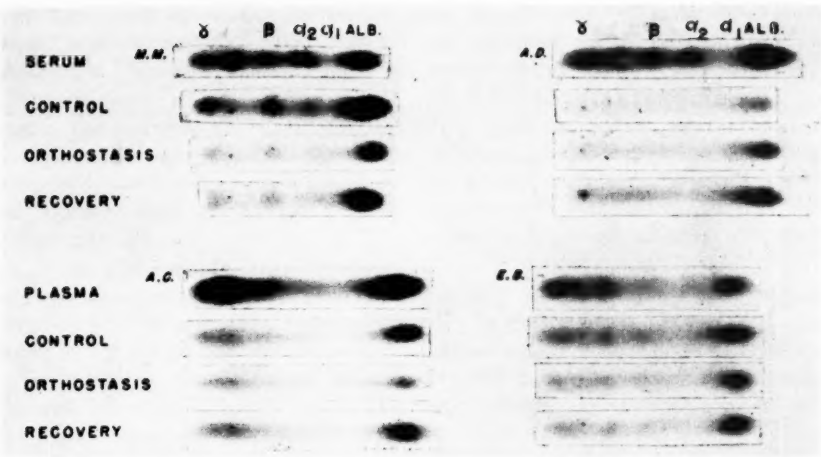


FIG. 3. PAPER ELECTROPHORETIC PATTERNS OF URINARY PROTEINS DURING CONTROL ORTHOSTASIS AND RECOVERY

No change from the control pattern was detectable in the urinary protein mixture during or after orthostasis, other than that attributable to dilution of all fractions, as a result of "washing out" the bladder. Hence, all protein fractions appeared to participate in the reduction of protein excretion; none was selectively affected to a demonstrably greater or less extent than the others.

## DISCUSSION

In normal man, the assumption of the upright position sets in train widespread vasoconstriction in which the renal vasculature participates (18, 19). The present study demonstrates the persistence of such an intra-renal vasoconstrictive response in severe acute or chronic disease of the kidney. Undoubtedly, the magnitude of the observed response was exaggerated to some extent by vasomotor instability and inadequate maintenance of arterial pressure in patients who were ill and bedfast. In almost every instance, effective renal plasma flow decreased relatively more than the mean brachial arterial pressure shortly after tilting or standing upright. Glomerular filtration usually fell in proportion to renal plasma flow. Urine flow declined and the urinary protein concentration tended to increase, thus accounting for the clinical observation of "postural proteinuria" in the course of chronic renal disease. However, total protein excretion usually decreased to about the same extent as filtration. Hence, intra-renal vasoconstriction appeared to affect protein output by reducing protein filtration rather than by changing glomerular capillary permeability.

Since the glomerular filtration rate and the maximal tubular reabsorptive capacity for glucose ($T_{mg}$) appeared to decrease proportionately, the fall in filtration may be ascribed to a diminution in the area of the filtering surface, presumably as a result of cessation of function in a fraction of the nephrons. A similar fall in filtration and $T_{mg}$ has also been observed in normal subjects during orthostasis (20). The glomerular injury in the diseased kidney is notably diverse (21), ranging from complete obliteration of some units to absence of demonstrable anatomical alteration in others. Hence, the fact that protein excretion, filtration rate, and glucose $T_m$ all appeared to decrease to more or less the same extent in this study strongly suggests that vasoconstriction within the diseased kidney during orthostasis does not affect any category of glomeruli preferentially. The selective elimination of the less permeable glomeruli from function would be expected to reduce albumin excretion more than globulin excretion, if it may be assumed that glomerular permeability under these circumstances is primarily a function of molecular size. Albumin excretion would then decrease more and globulin excretion less than total protein output.

On the other hand, selective elimination of the more permeable glomeruli would have the opposite effect of increasing albumin output relative to globulin. However, paper elctrophoresis failed to disclose any detectable change in urinary protein pattern during or after orthostasis, thus providing further evidence that the glomerular population which remains in function is a random sample of the total in operation prior to orthostasis.

This study was not designed to test critically the question of the relative contributions of glomerular and tubular derangements in the mechanism of proteinuria. It has been assumed throughout that changes in glomerular permeability play a predominant role, but the inferences based on this assumption, which are set forth here, apply with equal force if a defect in tubular protein reabsorption is of importance. The variability in the character of protein loss from patient to patient and the lack of a clear-cut pattern of predictable change throughout the course of renal disease noted in these data (Table I and Figure 3) suggest that factors other than the shape and dimensions of the plasma protein molecules may determine to some extent their escape into the urine. Thus, in one subject in terminal uremia (A. D., Figure 3) the urinary protein was almost entirely albumin, whereas in another with much less functional impairment (M. M.) the urinary protein pattern closely resembled that of the patient's plasma and contained a large amount of globulin. On the whole, however, total protein loss was correlated with the available filtering surface (Table I), decreasing as filtration declined with advance in the disease process. Moreover, protein escape apparently decreased less rapidly than filtration during the course of disease (Table I). As a result, the protein output-inulin clearance ratio tended to be high when the kidney was severely damaged, conforming with the widely-accepted view that glomerular permeability tends to increase with progression of disease (3, 6, 7, 8, 22, 23).

## SUMMARY

An investigation of the effect of the renal hemodynamic adjustments during orthostasis on protein excretion was made in nineteen patients with acute and chronic renal disease. Glomerular filtration rate (inulin clearance) and effective renal plasma flow (p-aminohippurate clearance) decreased almost immediately after assumption of the upright position in nearly every instance, apparently as a result of intra-renal vasoconstriction. Although the urinary protein concentration often increased under these circumstances the total protein output fell to approximately the same extent as filtration. The maximal rate of tubular reabsorption of glucose (glucose $T_m$) decreased with filtration in six determinations, indicating that cessation of glomerular activity in a proportion of nephrons accounted for the fall in filtration. Filtration, protein output, and glucose $T_m$ tended to fall proportionately despite the diversity inherent in a diseased nephron population. It was concluded, therefore, that the population of nephrons remaining in operation during orthostasis did not differ substantially from the total. All constituents of the urinary protein mixture seemed equally affected since no obvious changes in urinary protein patterns obtained by paper electrophoresis were observed during or after orthostasis. This finding was interpreted as further evidence of a diffuse and relatively uniform vasoconstrictive response that did not significantly alter glomerular capillary permeability.

## ACKNOWLEDGMENTS

We wish to express our gratitude to Mrs. Katherine T. Baker, Mrs. Joan H. Banfield, Miss Barbara Jones, Miss Phyllis Kallenberg, and Mrs. Lottie F. Klayman for their efficient and valuable technical assistance.

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# PREGNANCY AND ADRENOCORTICAL FUNCTION: ENDOCRINE STUDIES OF PREGNANCY OCCURRING IN TWO ADRENAL-DEFICIENT WOMEN.[1]

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(Submitted for publication February 23, 1954; accepted July 6, 1954)

It has been suggested by Jailer and Knowlton (1) that when adrenal-deficient women become pregnant, they exhibit "simulated adrenal cortical activity" attributable to an extra-adrenal source of "adrenal cortical-like" hormone. Observations which can be interpreted as lending support to this view may be summarized as follows:

1. Pregnancy has been reported to prolong the survival of adrenalectomized animals (2–4).
2. Urinary 17-ketosteroid elimination (as estimated by the Zimmermann reaction) rises during the latter months of pregnancy in patients with Addison's disease (1, 5, 6) as well as in normal women (7).
3. Urinary excretion of neutral reducing lipids rose to the normal range late in pregnancy in one patient with Addison's disease (1).
4. A decrease of circulating eosinophils four hours following the injection either of epinephrin or of corticotrophin was observed on three occasions in one Addisonian patient during pregancy and not thereafter (1).

The occurrence of pregnancy in two women rendered adrenal-deficient by surgery because of severe hypertensive disease has provided an opportunity to conduct certain studies, reported in this communication, which bear upon the question.

[1] This investigation was supported in part by research grants H-1419 and H-922 from the National Heart Institute, U. S. Public Health Service; by a grant from the National Research Council of Canada; and by grants from the Squibb Institute for Medical Research, Eli Lilly and Company, Ciba Pharmaceutical Products, Inc., Merck and Company, Inc. and the Publicker Industries Nutritional Fund.

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## METHODS

Sodium and potassium were determined on a flame photometer with internal lithium standard (8); creatinine by the procedure of Bonsnes and Taussky (9); urea by Karr's method (10). Urinary 17-ketosteroids were estimated by the Holtorff-Koch modification (11) of the Zimmermann reaction. The measurements were of total neutral 17-ketosteroids, which would be expected to be higher than values for the ketonic fraction commonly reported. Neutral reducing lipids were estimated using the phosphomolybdate reaction according to Heard, Sobel, and Venning (12), and glycogenic corticoids by the bioassay technique of Venning, Kazmin, and Bell (13). Values reported as "$pH_1$ hydrolyzed" represent determinations on extracts of urine acidified with hydrochloric acid and kept at $pH_1$ for one hour. "Glucuronidase hydrolysis" signifies that the extracted material had initially been subjected to hydrolysis for 48 hours at pH 4.5 in the presence of $\beta$-glucuronidase (derived from spleen) in a concentration of 100 units per milliliter, and subsequently to acid hydrolysis as described above.

In the tables, excretion of urinary steroids is generally given both uncorrected and corrected for urinary creatinine excretion. The latter method of expression was thought preferable where many of the urine collections were made outside the hospital, as in the case of the data presented in Figure 1; in Figure 2, uncorrected data are charted because creatinine analyses were not available for all samples. Values for glycogenic corticoid excretion are uncorrected for urinary creatinine excretion, and represent cortisone equivalents.

Eosinophils were enumerated by the method of Manners (14).

### *Case reports*

1. *S. C.*, H.U.P., Ob. 43769, was a 36-year-old colored female who had bilateral thoracolumbar sympathectomy and subtotal adrenalectomy performed for hypertension by Dr. Harold A. Zintel in three stages in June 1949 and March 1950. Her case has been reported in detail elsewhere (15). She became pregnant in August 1951. Gestation was uneventful, and she continued to take no steroid save for an occasional 12.5 or 25 milligrams of

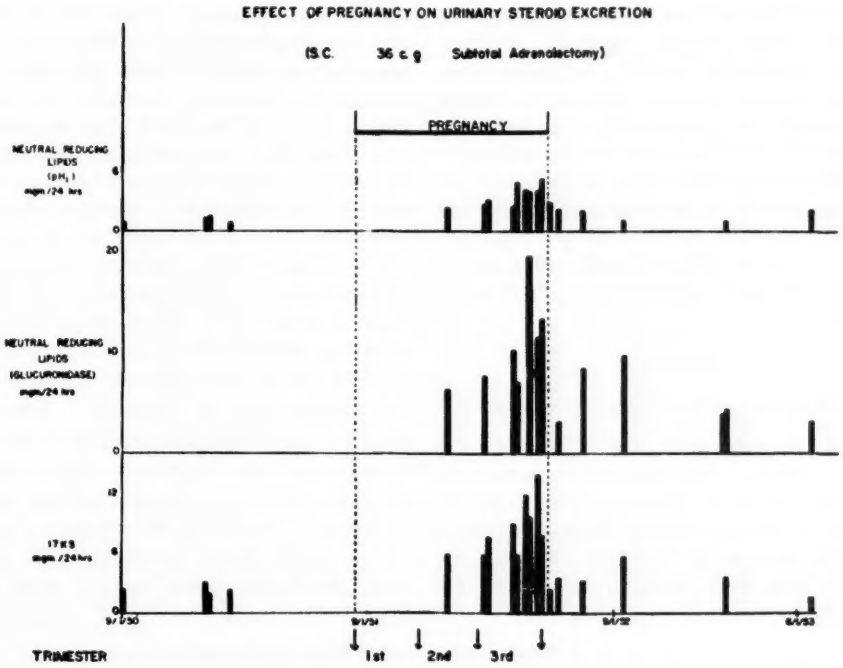


FIGURE 1.

cortisone. The blood pressure was little affected by pregnancy, remaining throughout in the range of 115–140/80–100. On May 19, 1952, she was delivered by Cesarean section, performed by Dr. George Hoffman, of a normal male infant weighing 1280 grams. Cortisone was administered at this time, and there were no post-partal complications. Mother and child remain in good health.

2. *B. L.*, H.U.P., Ob. 44812, was an 18-year-old white female followed by us since January 1951. Her complaint, at that time, was severe and progressive headache of six months' duration, accompanied by dizziness, blurring of vision, nausea, and vomiting. Blood pressure was 200–240/140–180, and retinopathy, Keith-Wagner Grade IV, was present. Phenolsulfonphthalein excretion was 15 per cent in 15 minutes, 70 per cent in two hours.

Bilateral subdiaphragmatic sympathectomy and splanchnicectomy, left 95 per cent adrenalectomy, and right total adrenalectomy were performed as a two-stage procedure by Dr. Zintel on January 27 and February 6, 1951. After operation, her symptoms were relieved and the retinopathy reverted to Grade I and II, but her blood pressure remained high (165–200/120–140). She required no replacement therapy, and metabolic studies revealed the presence of measurable adrenal cortical reserve.

The patient was married in 1952, and in early July 1952 became pregnant. She was admitted to the hospital in September 1952, at which time the site of the left adrenal was explored and an adrenal remnant weighing 1.1 Gm. was removed. She was hospialized throughout much of the remainder of her pregnancy and required a minimum of 25 mgm. of oral cortisone daily at all times. She felt well but her blood pressure remained elevated throughout her pregnancy. On January 20, 1953, in the 29th week of pregnancy, the patient went into spontaneous labor and was delivered by Dr. Michael Newton of a living female infant weighing 800 grams, which, however, died of prematurity after three days. The post-partal course was uneventful and the patient, at the present time, remains asymptomatic, though her blood pressure is high (210–230/140–155). She continues to require a minimum of 25 milligrams of oral cortisone daily.

## COMMENT

It is clear that both our patients had gross adrenal deficiency.[3] B. L. may have had total deficiency, although we know of no practical method of ascertaining the complete absence of functioning adrenal cortical tissue. S. C. is certainly adrenal-deficient, as indicated by both melanosis and by her susceptibility to the precipitation

[3] For convenience the term *deficiency* as used in this communication, refers to lack of adrenal cortical tissue, of whatever degree; *insufficiency* indicates the physiologic consequences, of whatever gravity, of lack of available hormone in relation to need.

of adrenal insufficiency; but she probably has some functioning cortical tissue, since she requires little or no substitution therapy. Repeated study of 24-hour urinary sodium elimination during pregnancy in the two patients while they were taking self-selected diets indicated that the difference between the patients with respect to their need for substitution therapy is probably not attributable to any important difference in their salt consumption: S. C. excreted 228 ± 42 milliequivalents of sodium per 24 hours, and B. L. 186 ± 23 milliequivalents.

## RESULTS

### *Effect of pregnancy upon urinary steroid excretion*

*Neutral reducing lipids and 17-ketosteroids (Figures 1 and 2).* The quantities of neutral 17-ketosteroids excreted following pregnancy by B. L., and of neutral reducing lipids and neutral ketosteroids excreted by S. C. in the non-pregnant state, were both distinctly lower than the mean values for normal women and for hypertensive women obtained in this laboratory. During pregnancy excretion of these substances by both women was augmented, the values for neutral reducing lipids falling within the range of values which have been observed at corresponding times in a small number of observations of normal pregnancy in this laboratory. The increases were unrelated to hormone therapy, for in one patient (S. C. [Figure 1]) no hormone was given within 48 hours prior to the beginning of any of the collections, while in the other (B. L. [Figure 2]) cortisone administration was kept substantially constant (25 to 37 milligrams daily) during and immediately prior to collections. The effect of pregnancy upon urinary steroid excretion in B. L. is somewhat more impressive when correction is made for urinary creatinine excretion, though by no means as striking as the influence of pregnancy upon urinary streoid excretion in S. C. However, the highest values in S. C. were observed

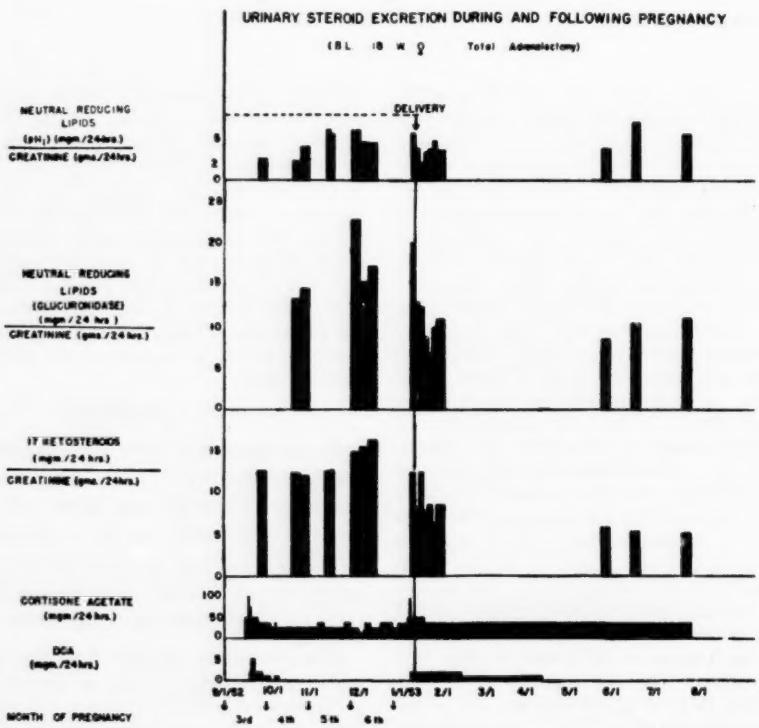


FIGURE 2.

TABLE I

*Urinary excretion of glycogenic corticoids by adrenal-deficient women during and following pregnancy*

| Patient | Date | Medication* mgm./24 hr. | Month of pregnancy | Glycogenic corticoids μgm./24 hr. |
|---|---|---|---|---|
| S. C. | 4/9–11/52 | 0 | 8th | 114 |
| | 4/28–30/52 | 0 | 9th | 130 |
| | 9/17–19/52 | 0 | Post-partum | 26 |
| | 2/9–11/53 | 0 | Post-partum | 41 |
| | 6/20–22/53 | 0 | Post-partum | 34 |
| | 6/22–24/53 | ACTH 60 | Post-partum | 46 |
| | 6/24–26/53 | ACTH 60 | Post-partum | 38 |
| | 6/26–28/53 | ACTH 60 | Post-partum | 50 |
| | 6/28–30/53 | ACTH 60 | Post-partum | 42 |
| B. L. | 10/22–24/52 | E 25 | 4th | 88 |
| | 12/2–4/52 | E 25 | 6th | 151 |
| | 5/31 to 6/2/53† | E 37 | Post-partum | 76 |
| | 6/2–10/53† | E 37 ACTH 60 | Post-partum | 42 |
| | 6/25–27/53 | E 37 | Post-partum | 45 |
| | 7/27–29/53 | E 37 | Post-partum | 23 |

* E: Cortisone acetate (oral), supplied by Dr. Elmer Alpert of Merck and Company. ACTH: H. P. Acthar Gel (intramuscular), supplied by Dr. C. J. O'Donovan of the Armour Laboratories.

† Hydrolyzed at $pH_1$ only. All other samples subjected to initial hydrolysis with β-glucuronidase followed by hydrolysis at $pH_1$.

during the last six weeks of pregnancy, and comparable specimens were not obtained in B. L. owing to her premature delivery. Whether or not the creatinine correction is applied, a progressive increase of urinary steroid excretion beginning as early as the second trimester is observable in both patients during pregnancy.

*Glycogenic corticoids* (*Table I*). Material capable of causing glycogen deposition in the liver of the fasted adrenalectomized mouse was excreted in the urine of both patients at all times. Following pregnancy, the values have been of the order of magnitude found in patients with Addison's disease on minimal replacement therapy, and in B. L. the excretion of this material may be attributable, in part at least, to oral cortisone ingestion. During the latter half of pregnancy both women were excreting three to four times this quantity, and at these periods the values were in the range found in patients with Cushing's disease. These values fall close to the mean of those observed in normal pregnancy at corresponding times as determined in the same laboratory (*7*).

### *Physiological studies during pregnancy*

*Response to intravenous corticotrophin* (*Table II*). Repeated attempts were made to obtain evidence of an adrenal-like response to 24 or 48-hour corticotrophin tests. Either 10 or 20 U.S.P. units in glucose or saline solution were administered by intravenous infusion over an eight-hour period, in order to supply a near maximum stimulating dose (16). Adrenal-like response was evaluated in terms of 24 or 48-hour excretion of neutral reducing lipids (using two methods of hydrolysis), and of 17-ketosteroids, comparing the experimental periods (reckoned from the beginning of the 8-hour infusions) with control values, usually those for the period immediately preceding the test infusion; and also by comparing the eosinophil counts prior to and at the termination of the infusion.

Responses to the infusions of corticotrophin uniformly were trivial or lacking in S. C. The normal eosinophil decrease at the end of such an 8-hour infusion amounts to a minimum of 90 per cent and the average increment in 24-hour urinary steroid excretion (*i.e.*, the mean increment of neutral reducing lipids determined following hydrolysis at $pH_1$ and by β-glucuronidase and of 17-ketosteroids) amounts to a minimum of 6 milligrams (17). A significant reduction (17) of circulating eosinophils was never seen in either patient. In two of the tests in B. L., steroid out-

TABLE II

*Effect upon urinary steroid excretion and circulating eosinophils of 8-hour intravenous corticotrophin infusions during pregnancy in adrenal-deficient women*

| Pt. | Date | Day of pregnancy | ACTH* *USP units* | Creatinine *gm./24 hr.* | Steroids (*mgm./24 hr.*) Neutral reducing lipids pH1 | Steroids (*mgm./24 hr.*) Neutral reducing lipids Glucuronidase | Steroids (*mgm./24 hr.*) 17-KS | Steroids (*mgm./24 hr.*) / Creatinine (*gm./24 hr.*) Neutral reducing lipids pH1 | Steroids (*mgm./24 hr.*) / Creatinine (*gm./24 hr.*) Neutral reducing lipids Glucuronidase | Steroids (*mgm./24 hr.*) / Creatinine (*gm./24 hr.*) 17-KS | Eosinophils Before ACTH *per mm.*³ | Eosinophils After ACTH *per mm.*³ | Eosinophils Change *per mm.*³ | Eosinophils Change % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1952 | | | | | | | | | | | | | |
| S. C. | 2/27–28 | 198 | 0 | .97 | 3.3 | X | 7.6 | 3.4 | X | 7.8 | | | | |
| | 2/29–3/1 | 199 | 10 | .94 | 2.1 | 7.0 | 6.9 | 2.2 | 7.5 | 7.3 | 125 | 80 | − 45 | −36 |
| | 4/1–2 | 231 | 0 | 1.00 | 2.5 | 10.2 | 9.1 | 2.5 | 10.2 | 9.1 | | | | |
| | 4/4–5 | 232 | 10 | 1.10 | 2.9 | 9.0 | 11.8 | 2.6 | 8.2 | 10.7 | 84 | 76 | − 8 | −10 |
| | 5/11–12 | 272 | 0 | 1.02 | 4.2 | 11.5 | 13.8 | 4.1 | 11.3 | 13.5 | | | | |
| | 5/13–14 | 274 | 20 | 1.08 | 4.1† | 19.6† | 10.6† | 3.8† | 18.1† | 9.8† | 47 | 55 | + 8 | +17 |
| | 5/14–15 | 275 | 20 | 1.27 | 4.0 | 15.7 | 10.9 | 3.1 | 12.4 | 8.6 | 41 | 34 | − 7 | −17 |
| | 7/15–16‡ | | 0 | .75 | 2.0 | 8.2 | 3.2 | 2.7 | 10.9 | 4.3 | | | | |
| | 7/16–17‡ | | 10 | 1.33 | 2.5 | 6.1 | 2.6 | 1.9 | 4.6 | 2.0 | 45 | 54 | + 9 | +20 |
| B. L. | 9/25–26 | 117 | 0 | .55 | 1.4 | X | 6.9 | 2.5 | X | 12.6 | | | | |
| | 10/2–3 | 123 | 10 | .68 | 4.8 | 13.6 | 6.0 | 7.1 | 20.0 | 8.8 | 242 | 166 | − 76 | −31 |
| | 10/3–4 | 124 | 10 | .64 | 4.1 | 12.7 | 11.6 | 6.4 | 19.8 | 18.2 | 233 | 128 | −105 | −45 |
| | 10/29–30 | 150 | 0 | .53 | 2.1 | 7.7 | 6.4 | 4.0 | 14.5 | 12.1 | | | | |
| | 10/30–31 | 151 | 10 | .56 | 3.0 | 11.7 | 7.3 | 5.4 | 20.8 | 13.0 | 179 | 230 | + 51 | +28 |
| | 10/31–11/1 | 152 | 10 | .55 | 3.7 | 10.6 | 7.5 | 6.7 | 19.3 | 13.6 | 165 | 175 | + 10 | + 6 |
| | 12/4–5 | 186 | 0 | .79 | 3.5 | 13.5 | 12.9 | 4.4 | 17.1 | 16.3 | | | | |
| | 12/8–9 | 190 | 10 | .75 | 2.4 | X | 11.3 | 3.2 | X | 15.0 | 144 | 166 | + 22 | +15 |

* Corticotrophin supplied by Dr. E. C. Vonder Heide of Parke, Davis and Company.
† Oral cortisone acetate 25 milligrams given on morning of 5/13.
‡ Two months post-partum.

put was somewhat increased on the days when corticotrophin was administered; but the increases are small and of questionable significance, and no effect of corticotrophin is observable on the occasions of the third test.

*Precipitation of acute adrenal insufficiency (Figures 3, 4, and 5).* Evidence of acute adrenal insufficiency was obtained in both patients during pregnancy. B. L. was hospitalized for much of the gestational period, and an effort was made, for therapeutic reasons, to supply no more than the minimum required replacement therapy. Symptoms of incipient acute adrenal insufficiency appeared promptly whenever an attempt was made to reduce the daily ration of cortisone acetate below 25 milligrams per day. In Figure 3 are shown data indicating that adrenal insufficiency promptly followed reduction of exogenous cortisone to 12.5 milligrams daily; immediately prior to hormone reduction she was excreting glycogenic corticoids in the urine in the amount of 151 micrograms per 24 hours (Table I). Frank symptomatic and chemical evidence of early acute adrenal insufficiency later appeared, in spite of the provision of additional exogenous hormone, in the wake of a dental infection followed by a tooth extraction (Figure 4).

S. C. was followed as an outpatient during most of her pregnancy. She was admitted to the Metabolic Unit during the seventh month of pregnancy, in the course of which evidence was obtained that acute adrenal insufficiency could readily be precipitated by dietary sodium restriction (Figure 5). Urinary glycogenic corticoid was first determined six weeks later, but the excretion of neutral reducing lipids and 17-ketosteroids was already elevated (Figure 1).

*Response to corticotrophin following pregnancy (Tables I and III).*

A daily injection of potent, long-acting corticotrophin was given for eight days to both patients well after the post-partal period in an attempt to ascertain whether they harbor residual

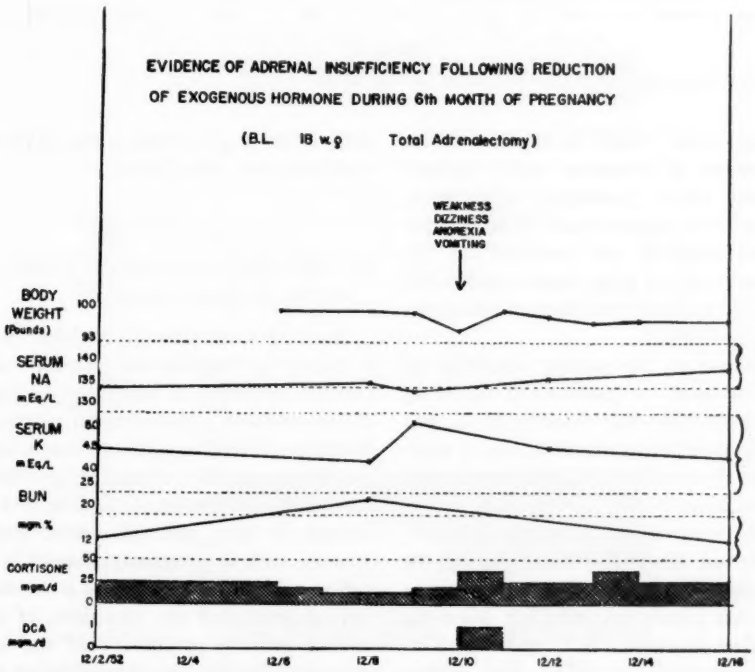


FIGURE 3.

Dotted lines represent the normal range for our laboratory.

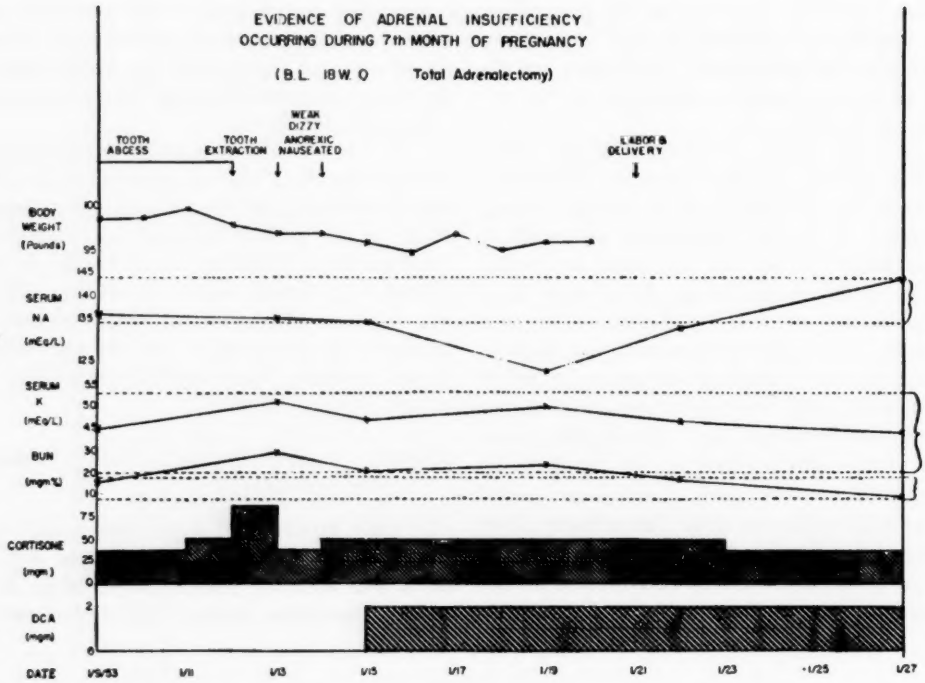


FIGURE 4.

Dotted lines represent the normal range for our laboratory.

adrenal cortical tissue which is capable of increasing its output of hormone under intense, continuous, and rather prolonged stimulation. One control and four experimental 48-hour urine collections were obtained and analyzed for 17-ketosteroids, neutral reducing lipids (following hydrolysis at $pH_1$ and also with $\beta$-glucuronidase), and glycogenic corticoids.

A small increase in the urinary excretion of neutral reducing lipids as determined following glucuronidase hydrolysis was noted in B. L. following corticotrophin administration; and a similar increase was observed in S. C., the increase being more convincing if the uncorrected steroid values are used. Conversely, an increment of urinary 17-ketosteroid observable in B. L. on the basis of the values corrected for urine creatinine excretion becomes rather unconvincing when the uncorrected values are used. S. C. showed no increase of 17-ketosteroid excretion, and neither patient increased her output either of neutral reducing lipids as determined following acid hydrolysis or of glycogenic corticoids in response to corticotrophin stimulation.

## DISCUSSION

### *Do adrenal-deficient women exhibit adrenal-like activity during pregnancy?*

It would seem desirable to define precisely what is meant by "adrenal cortical-like" activity. Increased excretion of neutral reducing lipids and 17-ketosteroids (Zimmermann reaction) certainly indicates adrenal cortical activation under certain conditions, notably adrenal stimulation by corticotrophin; yet the simple finding of increased excretion of these materials under special circumstances, such as pregnancy, cannot be assumed to reflect adrenal activity unless it is reasonably certain at least that the secretions of other glands cannot provide precursors of the urinary substances giving the phosphomolybdate and Zimmermann reactions. For example, an increase in the urinary excretion of 17-ketosteroids derived from

TABLE III

*Effect of depot (intramuscular) corticotrophin upon urinary steroid excretion and circulating eosinophils in adrenal-deficient women following pregnancy*

| Pt. | Date | ACTH* *USP units* | Urine creatinine *Gm./24 hr.* | Steroid (*mg./24 hr.*) Neutral reducing lipids pH1 | Steroid (*mg./24 hr.*) Neutral reducing lipids G'dase | Steroid (*mg./24 hr.*) 17-KS | Steroid (*mg./24 hr.*) / Creatinine (*Gm./24 hr.*) Neutral reducing lipids pH1 | Steroid / Creatinine G'dase | Steroid / Creatinine 17-KS | Eosinophils *per mm.*³ | Eosinophils Date |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1953 | | | | | | | | | | 1953 |
| S. C.† | 6/20–22 | 0 | .73 | 2.2 | 3.1 | 1.6 | 3.0 | 4.2 | 2.2 | 45 | 6/22 |
| | 6/22–24 | 60 | 1.08 | 2.0 | 4.5 | 3.8 | 1.9 | 4.2 | 3.5 | | |
| | 6/24–26 | 60 | .98 | 2.5 | 7.9 | 3.4 | 2.6 | 8.1 | 3.5 | | |
| | 6/26–28 | 60 | 1.30 | 3.5 | 7.5 | 2.6 | 2.7 | 5.8 | 2.0 | 64 | 6/29 |
| | 6/28–30 | 60 | 1.25 | 3.0 | 6.0 | 2.2 | 2.4 | 4.8 | 1.8 | 55 | 6/30 |
| B. L.‡ | 5/31–6/2 | 0 | .98 | 3.7 | 8.4 | 5.6 | 3.8 | 8.6 | 5.7 | 155 | 6/2 |
| | 6/2–4 | 60 | .85 | 3.4 | 11.6 | 5.1 | 4.0 | 13.7 | 6.0 | 255 | 6/4 |
| | 6/4–6 | 60 | .74 | 2.9 | 10.8 | 4.7 | 3.9 | 14.6 | 6.4 | 171 | 6/5 |
| | 6/6–8 | 60 | .92 | 3.2 | 13.2 | 7.0 | 3.5 | 14.3 | 7.6 | 137 | 6/6 |
| | 6/8–10 | 60 | .80 | 2.7 | 12.6 | 6.4 | 3.4 | 15.8 | 8.0 | 160 | 6/10 |

* H. P. Acthar Gel, supplied by Dr. C. J. O'Donovan of the Armour Laboratories.
† Thirteen months post-partum.
‡ Four and one-half months post-partum. Patient received, throughout the period shown, 37 milligrams of oral cortisone acetate daily, supplied by Dr. Elmer Alpert of Merck and Company.

an interstitial cell tumor would neither reflect adrenal activity nor imply the secretion of compounds possessing the characteristic structural or biologic properties of the alpha-ketolic hormones of the adrenal cortex.

It is proposed, therefore, to understand by "adrenal-like" activity the secretion of compounds with physiological properties supposed to be possessed exclusively or at least predominantly by the C-21 alpha-ketolic steroid hormones of the adrenal

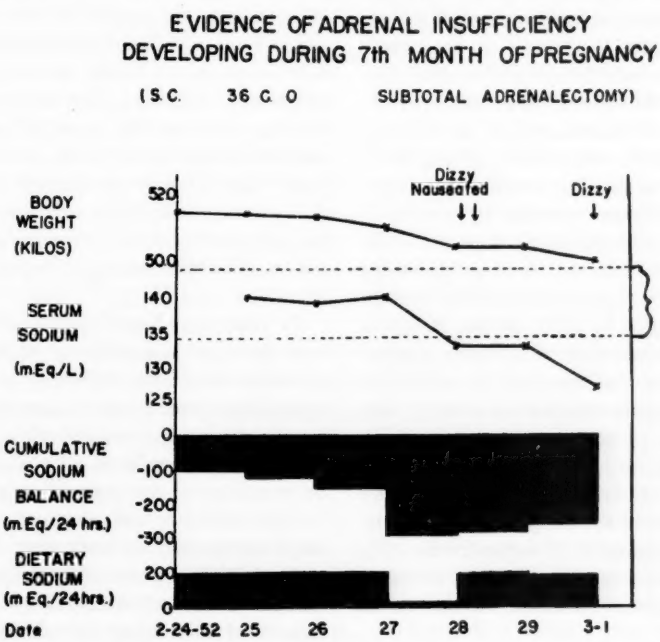


FIGURE 5.

Dotted lines represent the normal range for our laboratory.

cortex. It would appear that any attempt to establish the development of adrenal cortical-like activity during pregnancy in adrenal-deficient women, in this sense, must start with one or more of the three following findings: 1) augmented recovery from biological fluids, during pregnancy in such women, of compounds which can be shown to have the chemical structure or characteristic physiologic activity of C-21 alpha-ketolic steroids, or else of compounds which can be derived from such steroids and from no others; 2) demonstration of a temporary capacity to respond during pregnancy to corticotrophin administration in terms of acceptable indices of augmented excretion of C-21 alpha-ketolic steroids; 3) demonstration of conspicuous protection, during the pregnant state, against the development of acute adrenal insufficiency.

1) The data (Figures 1 and 2) confirm the observation of others that adrenal-deficient women excrete an increased quantity of 17-ketosteroids and of neutral reducing lipids in the urine during pregnancy. However, in view of the non-specificity of the Zimmermann and phosphomolybdate reactions, and of the augmentation and diversification of the function of at least several of the endocrine organs during pregnancy, it is exceedingly difficult to exclude the possibility that hormones other than C-21 alpha-ketolic steroids elaborated by the ovary or placenta might serve as the precursors for urinary compounds giving these reactions. One of us (7) has specifically pointed out that the Zimmermann reaction, by which 17-ketosteroids are usually estimated, is given by the pregnanolones, which derive from progesterone and appear in greatly augmented quantity in pregnancy urine, as well as by other 3- and 20-ketosteroids. It is therefore open to question whether the increased urinary elimination of substances giving the Zimmermann reaction necessarily indicates the development of adrenal-like activity in pregnancy. Indeed, no increase in 17-ketosteroid excretion is commonly found in normal pregnancy when the more specific antimony trichloride reaction is used to estimate 17-ketosteroids (7), though in one Addisonian who became pregnant both methods indicated increased 17-ketosteroid elimination (6).

If the increased urinary excretion of neutral reducing lipids during pregnancy reflects an increased secretion of C-21 alpha-ketolic steroids, one might expect that the urinary values for neutral reducing lipids would parallel those for glycogenic corticoids throughout pregnancy; but the parallelism is not impressive in normal pregnant women (7). Furthermore, the phosphomolybdate reaction, which was used for the determination of neutral reducing lipids in our studies as well as in those of Jailer and Knowlton, is not specific for C-21 alpha-ketolic steroids; it is given, for example, by any substance possessing an alpha-beta unsaturated 3-ketone group (18), which is present in progesterone. It is true that progesterone has never been found in urine, and that the ketone function has been reduced in its known urinary derivatives. These, however, represent less than 20 per cent of its degradation products (19), and it is entirely possible that hitherto unidentified metabolites of progesterone could be responsible, in some part, for the increase in the excretion of neutral reducing lipids observed in these patients.

The glycogenic corticoids, however, can scarcely be supposed to derive from any source other than the 11-oxygenated C-21 alpha-ketolic steroids characteristically present in the secretion of the adrenal cortex. The increased elimination of these substances in the urine of our patients during pregnancy (Table I) therefore constitutes convincing evidence for increased endogenous production of such compounds, unless it can be supposed that a higher proportion of secreted cortical hormone reaches the urine in active form during pregnancy, owing to some alteration during that state of the usual pathways of steroid metabolism.

2) Jailer and Knowlton (1) observed a 40 per cent decrease in circulating eosinophils on three occasions four hours following a test injection of epinephrin (twice, subcutaneously) and corticotrophin (once, intramuscularly) during the latter part of pregnancy in an adrenal deficient woman; on repetition of the tests following pregnancy, no similar decrease was observed. These findings were interpreted as indicating a temporary capacity in the pregnant adrenal-deficient woman to exhibit adrenal or adrenal-like response to agents capable of stimulating the adrenal cortex.

A 50 per cent reduction of circulating eosinophils (20) has generally been taken to represent

the minimum decrease constituting a positive response to the four-hour ACTH test; and a recent statistical study (21) locates the minimum normal decrease at 60 per cent. An eosinophil decrease of 40 per cent in the four-hour ACTH test may well fall within the range of changes which can be ascribed to the combined effects of random fluctuations of the eosinophil count and of enumeration error. A similar objection may also be urged against the significance attached to the eosinophil responses to epinephrin; moreover, subsequent work has indicated that epinephrin may depress the eosinophil count by a mechanism other than adrenal cortical activation (22). In both of our patients, near maximal stimulation with intravenous corticotrophin failed to elicit evidence of any consequential adrenal-like response during pregnancy (Table II).

3) Available reports of the effect of pregnancy upon the survival of adrenalectomized animals (2–4, 23–25) throw little light upon the possible development of adrenal-like activity in human pregnancy. Any prolongation of survival which might be observed as a result of pregnancy in the adrenalectomized animal might perhaps be attributed to the beneficial effects of certain sex hormones rather than to adrenal-like activity; for estrus in dogs and cats (26, 27) and progesterone in rats and ferrets (28–31) have been reported to prolong the survival of adrenal-deficient animals. Actually, however, while the survival of adrenalectomized bitches is reported (3) to be prolonged by pregnancy, a similar report for cats (2) could not be confirmed by other investigators (23, 24); and experiments in the white rat (4, 25) are also conflicting. The lack of agreement on this subject among physiologists, and the possibility of differences between species, appear to compel reliance upon clinical studies for the elucidation of the effect of pregnancy upon the physiology of human adrenal deficiency.

Death from adrenal insufficiency occurring in association with pregnancy was frequent in Addisonians before salt and hormone therapy were developed; and while the fatalities commonly followed the stress of delivery or abortion, it appears that the condition of the untreated Addisonian may deteriorate progressively throughout gestation (32). The literature dealing with pregnancy complicated by Addison's disease, which has been reviewed to 1950 (6, 33), furnishes no evidence that hormone requirement regularly decreases at any time during pregnancy, substitution therapy having been considered necessary or desirable throughout gestation in most cases. The first trimester with its associated vomiting is especially hazardous even to the steroid-treated Addisonian (34, 35), but also in the third trimester extra therapy may be required (6), and adrenal crisis has been reported to follow dietary salt reduction and feeding of potassium in the seventh month (36). The vulnerability of our patients to adrenal insufficiency during the latter part of pregnancy (Figures 3, 4, and 5) is thus in accord with the experience of others.

Evidence to suggest the development of adrenal-like activity during pregnancy in adrenal-deficient women, then, is confined essentially to the finding that the urinary excretion of steroids commonly used as indices of adrenal cortical secretion, and especially of steroids exhibiting the biological activity of 11-oxygenated alpha-ketolic steroids, is enhanced during pregnancy in such women. The central problem in interpreting the data is the reconciliation of the vulnerability to adrenal insufficiency of the adrenal-deficient pregnant women with the concurrent high values for urinary excretion of glycogenic corticoids associated with pregnancy in such women. Since adrenal insufficiency in the physiologic sense results from inadequacy of hormone in relation to need, its appearance in our patients means only that the quantity of hormone available to them at the time (endogenous plus exogenous) was inadequate to their needs. If pregnancy itself very greatly increases the requirement for adrenal hormone, the augmented urinary steroid excretion of glycogenic corticoids might faithfully reflect an increased secretion of C-21 alpha-ketolic steroids, the adrenal insufficiency representing an increase of need in excess of increase of supply of hormone. On the other hand, if pregnancy does not strikingly increase basal hormone requirement, one would have to infer that the urinary steroid excretion of glycogenic corticoids by these patients does not reliably reflect the availability of alpha-ketolic steroids, but rather an increase in the proportion of available (secreted plus exogenous) hormone recovered in the urine; for it is difficult to suppose that a secretion rate corresponding to that

found in Cushing's disease would not be more than adequate to protect against the mild stress of salt withdrawal in S. C.,[4] or the more considerable stress of dental infection and tooth extraction in B. L.; nor could one otherwise account at all for the need for exogenous hormone exhibited by B. L. during her pregnancy.

A definitive choice between these alternatives is probably not warranted at present; but some evidence can be urged in support of the view that increased excretion of urinary steroids during pregnancy in these women may be attributable to alterations of the metabolism of secreted or administered steroids rather than an increased rate of secretion. Dobriner, Lieberman, Rhoads, and Taylor (37) found that the two 11-oxygenated 17-ketosteroids normally present in the urine of nongravid women are excreted in much smaller quantity as pregnancy progresses. As these compounds are indubitably derived from 11-oxygenated alpha-ketols ("glucocorticoids") secreted by the adrenal cortex (38) their near disappearance from the urine in normal pregnancy would appear to suggest that pregnancy is associated with hindrance to the oxidative removal of the alpha-ketol side chain of adrenal steroids. A decrease in the proportion of available alpha-ketolic steroid disposed of in this fashion would be consistent with an enhancement of the proportion of available alpha-ketol appearing as such (*i.e.*, as neutral reducing lipids and glycogenic corticoids) in the urine.

Moreover clinical experience with B. L. is somewhat difficult to reconcile with the view that very large quantities of adrenal steroids were being secreted endogenously by this patient. As shown in Figure 3, reduction of exogenous cortisone from 25 to 12.5 milligrams daily resulted in the prompt appearance of manifestations of adrenal insufficiency. If her total basal hormone requirement amounted to about 25 milligrams per day, a reduction of 12.5 milligrams per day would be expected to result promptly in the appearance of adrenal insufficiency; but if her requirement (and supply) of cortisone equivalent were many times greater, it seems surprising that withdrawal of so relatively small a quantity would be followed so promptly by symptomatic and chemical signs of adrenal insufficiency.

It is true that normal pregnant women are reported (39) to exhibit an increased concentration of "17-hydroxycorticoids" in the blood. Whether a similar increase is observable in pregnant adrenal-deficient women is not known; but it may be pointed out that the finding does not in any case afford conclusive proof of hyperadrenocorticism during pregnancy. If, as seems possible, pregnancy results in inhibition of the oxidative removal of the alpha-ketolic side chain, secreted 17-hydroxy alpha-ketolic steroids might be expected to survive for a longer period (and, hence, to be present in higher concentration in the blood) in a form detectable by the Porter-Silber reaction, though loss of biologic activity might well result from reduction of ring A or changes elsewhere in the steroid molecule.

There is thus nothing to disprove, and a good deal of circumstantial evidence to justify the suggestion, that the urinary steroid excretion in adrenal-deficient pregnant women may not reliably establish that any consequential increase in the supply of adrenal-like hormone occurs in such women during pregnancy.

With regard to the urinary steroid data in our patients there appear, therefore, to be two possible alternative interpretations neither of which can be definitely ruled out[5] at present: 1) The

[4] We have forborne to interject into the discussion the further complication of a distinction between the "glucocorticoids" (11-oxygenated alpha-ketolic steroids), whose secretion alone is reflected in the glycogenic corticoids, and the "mineralocorticoids." Yet in the instance of adrenal insufficiency precipitated by curtailment of sodium intake (Figure 5) one might raise the objection that a very large secretion of glucocorticoids might fail to protect against salt deprivation. A need for "mineralocorticoids" cannot, however, be invoked to account for B. L.'s requirement for small quantities of *cortisone* during her pregnancy. Nor can a need for mineralocorticoids very plausibly explain the precipitation of adrenal insufficiency by infection and surgery in a patient receiving both desoxycorticosterone and cortisone (Figure 4); for the 11-17-oxygenated steroids are presumably the principal agents conferring protection against stresses of this kind.

[5] Some guidance in selecting the correct explanation might possibly be anticipated from a quantitative comparison of the steroid excretion data from normal pregnant women with those from adrenal-deficient pregnant women. Any such hope is disappointed, for the individual variation in the excretion of glycogenic corticoids (7) and of neutral reducing lipids at any time during

urinary steroid values reflect a greatly increased rate of secretion of adrenal or adrenal-like steroids during pregnancy, the increased availability of such steroids being ineffective in protecting against adrenal insufficiency because of a concomitant marked increase of the requirement for adrenal hormone; or 2) the urinary steroid values are misleading when accepted at face value as indicators of the rate of adrenal secretion during pregnancy; the high values for the chemically determined steroids may be partly ascribable to nonspecific chromogens, and the excretion of neutral reducing lipids and glycogenic corticoids may reflect an increase in the proportion of available hormone eliminated in the urine as alpha-ketols.

*Are any adrenal-like steroids which may be secreted during pregnancy by adrenal-deficient women necessarily of extra-adrenal origin?*

Even if one were to adopt the view that the augmented excretion of urinary steroid during pregnancy indicates increased secretion of alpha-ketolic steroids, the inference that such compounds must be secreted by some organ other than the maternal adrenal cortex will still not be firmly established until it is wholly clear that the latter organ cannot be their source. Even in the "totally adrenalectomized" patient the possibility of residual adrenal cortical tissue cannot be wholly dismissed; and in the individual rendered adrenal-deficient by subtotal adrenalectomy (17) as well as in the patient with Addison's disease (40) there is evidence that measurable though subnormal cortical function can be present.

We know of no evidence which can exclude the possibility that an adrenal remnant or rest might not be affected by pregnancy in such a fashion as to increase its output of adrenal hormone. It is true that a barely adequate adrenal remnant should be at all times under near-maximal stimulation by endogenous corticotrophin, according to current concepts of the homeostatic mechanism regulating adrenal cortical secretion (41), and our two patients exhibited little evidence of adrenal response to 24 and 48-hour corticotrophin tests during pregnancy (Table II). It is, however, conceivable that stimulation over months by corticotrophin hypothetically elaborated in large excess during pregnancy might result in actual hypertrophy or an adrenal remnant with consequent increase in the secretion of adrenal hormone. An attempt (Tables I and III) to simulate such a putative effect of pregnancy, conducted in our patients in the post-partal state by continuous stimulation over an 8-day period with depot corticotrophin, is at best suggestive of a modest response of the adrenal cortex to corticotrophin; a large increase of steroid excretion, such as had accompanied pregnancy, could not be reproduced. Certainly, however, the possibility of such activation during pregnancy under the influence of more prolonged stimulation by corticotrophin, or perhaps by some separate adrenal-growth-promoting factor (42), has not been excluded.

It seems most unlikely that the fetal adrenal makes any appreciable contribution of adrenal cortical hormone to the maternal organism during pregnancy. The newborn infant, whether born of a normal (43) or an adrenal-deficient mother (1), excretes only very small quantities of urinary steroids, including biocorticoids, and is resistant to corticotrophin stimulation (44). Jailer and Knowlton (1) suggested that the placenta might constitute an extra-adrenal source of "adrenal-like" hormone; and placentas of animal and of human origin have subsequently been reported to contain biologically active corticoid material (45, 46). However, there is no evidence to exclude the possibility that the material is simply stored there.

Since it is not possible to state with assurance that pregnancy causes increased secretion of C-21 alpha-ketolic steroids in adrenal-deficient women, nor that such secreted steroids cannot originate in maternal adrenal tissue, satisfactory proof of any extra-adrenal source of adrenal hormone is lacking at present.

From the point of view of practical management of the adrenal-deficient woman who becomes pregnant, it is imperative to emphasize the fact that gestation does not decrease the hazard of

pregnancy is very large, and the data are few; in consequence no significant comparison can be made. One can state only that available data do not permit a demonstration that the excretion of neutral reducing lipids and of glycogenic corticoids differ significantly in pregnant adrenal-deficient patients as compared with normal pregnant women. No data are available in our laboratory concerning the excretion of 17-ketosteroids during normal pregnancy.

acute adrenal insufficiency. The physiological studies conducted in our patients, as well as numerous reports in the literature, necessitate the conclusion that any extra C-21 alpha-ketolic steroids, of whatever source, which may become available to the severely adrenal-deficient woman in pregnancy are unable to prevent the prompt appearance of acute adrenal insufficiency at any time during pregnancy when specific therapy is withheld. While pregnancy of itself appears to have little effect upon hormone requirement, its complications and the stress of delivery will ordinarily require additional replacement therapy exactly as will any stress in a non-pregnant adrenal-deficient person.

## SUMMARY

1. Increased urinary elimination of 17-ketosteroids (Zimmermann reaction) and of neutral reducing lipids is regularly found during pregnancy in adrenal-deficient women. The increase is observable as early as the beginning of the second trimester, and is progressive to term.

2. Urinary excretion of glycogenic corticoids was increased three- to four-fold during the latter half of pregnancy in two adrenal-deficient women.

3. Neither patient was protected by the pregnant state against the development of acute adrenal cortical insufficiency, and no decrease in hormone requirement was noted during pregnancy in the one patient requiring substitution therapy.

4. Neither patient developed during pregnancy any striking capacity to respond to near-maximal stimulation by intravenous corticotrophin over a 24- or 48-hour test period.

5. More prolonged (8-day) stimulation with depot corticotrophin following pregnancy failed in both subjects to reproduce the large increases of urinary steroids which accompanied pregnancy.

6. The values for the urinary excretion of 17-ketosteroids, neutral reducing lipids and particularly glycogenic corticoids observed in adrenal-deficient pregnant women suggest that such women acquire during pregnancy an endogenous source of large quantities of C-21 alpha-ketolic steroid hormones; but it is possible, for reasons which have been discussed, that these commonly used indices of adrenal secretory activity become misleading during pregnancy, and that little or no increase of the secretion of alpha-ketolic steroids accompanies pregnancy in adrenal-deficient women.

7. If adrenal-deficient women do develop an enhanced endogenous supply of adrenal-like alpha-ketolic steroids when they become pregnant, pregnancy must increase their requirement for hormone together with the supply since such women remain vulnerable to adrenal insufficiency during gestation. The source of supply of any such steroids is apparently not the fetal adrenal; that any organ other than residual material adrenal tissue can be implicated awaits demonstration.

## ACKNOWLEDGMENTS

The authors wish to acknowledge the aid and support of Drs. William A. Jeffers and Carl Bachman in the conduct of these studies, and their kindness in reviewing the manuscript.

Technical assistance was rendered by Mrs. Irmgaard Landolt, Mr. Walter Applin, Mrs. Helen Bulaschenko, Mrs. Hana Conover, and Miss Katherine Macatsoris.

Through the kindness of Dr. Elmer Alpert, the Merck Company sustained hospitalization costs for B. L. throughout much of her pregnancy.

We are indebted to Dr. James Walker for the sodium analyses used for the construction of Figure 5.

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# STUDIES IN STEROID METABOLISM. XXVI. STEROID ISOLATION STUDIES IN HUMAN LEUKEMIA

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(Submitted for publication March 26, 1954; accepted July 15, 1954)

The normal and abnormal physiology of white blood cells can be characteristically influenced by the hormonal environment. Adrenocortical hormones administered as such, or secreted under stimulation by ACTH have been shown to influence the distribution, mass, and functional activity of lymphoid tissue (1–6). The course and incidence of leukemia in test animals are intimately related to alterations in adrenal or gonadal function (7–12) and the striking, albeit temporary, amelioration achieved by adrenocortical or adrenocorticotropic hormones in human leukemia, is well known (13–16). Although decreased urinary ketosteroids have been demonstrated in leukemia (17, 18), excretion of urinary corticoids has been generally unaltered (18). However, few data are available relative to specific gonadal or adrenal hormone metabolites in human leukemia.

We have applied, to the study of this problem, the precise methods of isolation, identification, and quantitation of individual urinary steroids developed within the past decade (19–23). The detailed ketosteroid patterns of six subjects with chronic lymphatic leukemia during control periods and of one subject with acute myelogenous leukemia during a control interval and while on ACTH therapy are reported. The results give evidence of decreased secretory activity of both gonads and adrenal glands in these subjects. The simultaneous development of refractoriness to ACTH therapy in one patient, with evidence of progressive adrenal dysfunction as measured by the urinary steroids, raises, in addition, an interesting question about the relation between clinical relapse in this disease, and the hormonal environment.

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## METHODS

The urinary ketosteroid patterns of six subjects with chronic lymphatic leukemia were studied. Five of these subjects were male (M45, B58, S59, H62, B68), and one was female (H55). In addition, the urinary ketosteroids of one subject with acute myelogenous leukemia (R29) were studied during the course of two successive therapeutic trials with ACTH. Metabolic balance studies were conducted during these periods and the results have already been reported (Subject R. R. [24]). All urine collections were short term (5 to 16 days) except for that of subject M45 (195 days). The urinary steroid conjugates were hydrolyzed immediately after collection, by a combination of methods employing enzymatic (beef liver $\beta$-glucuronidase), cold and/or hot acid hydrolysis (Methods A, B, C, or D [22]).

Neutral steroid extracts were separated into "ketonic" and "non-ketonic," and "$\alpha$-ketonic" and "$\beta$-ketonic" fractions by the usual techniques (19). The "$\alpha$-ketosteroid" fractions were further analyzed by adsorption and partition chromatography employing silica gel columns. Alumina and/or magnesium silicate columns were employed in certain fractionations. Individual steroid fractions were identified by infrared spectrometry and quantitated by the modified Zimmermann reaction.

The further details of the analytical procedures employed in these studies have already been described elsewhere (19, 22). Variable proportions of 3$\alpha$, 11$\beta$-dihydroxyandrostane-17-one and 3$\alpha$, 11$\beta$-dihydroxyetiocholane-17-one appeared as the $\Delta 9(11)$-analogs after vigorous acid hydrolysis. The values reported for the 11-hydroxylated compounds include these transformation products. Quantitative estimations of 17$\alpha$-hydroxypregnanolone were made by the Zimmermann reaction. Because the correction factor for this compound is high, the quantitative values may be seriously in error.

## RESULTS

The quantitative values for isolated and identified steroids only, are reported in the tables. The age follows the initial of each subject studied.

### *Chronic lymphatic leukemia*

*Subject M45:* This patient was a white man, admitted to the hospital with an eight-month history of marked weight loss, weakness, adenopathy,

scrotal and ankle edema and ascites. There was marked pallor on examination and adenopathy and splenomegaly were pronounced. The diagnosis of chronic lymphatic leukemia was established from marrow aspiration. Urine collection began two months after admission and continued for 195 days. The only therapy given during this collection period was the administration of repeated blood transfusions. Throughout the collection period, this subject was quite ill.

Urinary ketosteroid data are shown in Table I. The sum of the 11-desoxyketosteroids was well below average for this age group. The sum of the 11-oxygenated $C_{19}$ steroids was within normal limits.

*Subject H55:* This subject was a white woman who came to the hospital with a proved diagnosis of chronic lymphatic leukemia. She had been an X-ray technician for 25 years. Seventeen years previously at another hospital, radiation menopause had been induced for therapy of uterine fibroids. Except for lymphadenopathy, she was essentially well during the urine collection period.

The sum of the 11-oxygenated $C_{19}$ steroids was within the range observed in a series of normal women (25). The absence of androsterone and etiocholanolone from this subject's urine is very unusual.

*Subject B58:* This subject was a white man, admitted to the hospital with a two-year history of progressive fatigue. Examination revealed a high white blood cell count, hepato-splenomegaly and generalized adenopathy. The diagnosis of chronic lymphatic leukemia was established by marrow aspiration. Urinary steroids were studied during a 15-day control period preceding hormone therapy.

Levels of 11-oxygenated and 11-desoxy $C_{19}$ steroids during this control period, were considerably lower than those of normal men of similar age.

*Subject S59:* This subject was a white man, with a 12-month history of adenopathy, admitted to the hospital one year previous to the present study. The diagnosis of chronic lymphatic leukemia had been established at that time. This patient had received several forms of therapy during the year previous to study, including Amethopterin and X-ray, with little effect on his massive adenopathy and little decrease in the size of his liver or spleen. Urinary steroids were studied during a five-day control period preceding ACTH therapy.

The sums of both $C_{19}$ 11-oxygenated and 11-desoxysteroids were below the lowest limits observed in a series of normal men (22).

*Subject H62:* This subject was a white man, admitted to the hospital with a one-year history of weakness and weight loss and with a reportedly high white blood cell count. Clinical and laboratory examination established the diagnosis of chronic lymphatic leukemia. This subject was somewhat febrile during the control period. The influence of his febrile course upon his steroid pattern is open to question.

The sum of 11-oxygenated $C_{19}$ steroids was at

TABLE I

*Ketosteroid patterns in chronic lymphatic leukemia*

| Compounds | Leukemic patients | | | | | | Five male leukemics 45 to 68 years | Normal males 50 years and above |
|---|---|---|---|---|---|---|---|---|
| | *Milligrams per 24 hours* | | | | | | Average values | Average values |
| $C_{19}$ 11-oxysteroids | 1.8 | 1.5 | .4 | .3 | 1.2 | 1.3 | 1.0 | 1.8 |
| $C_{19}$ 11-desoxysteroids | .7 | — | .7 | .3 | .9 | 1.0 | .7 | 4.4 |
| 11-Ketoetiocholanolone | .3 | .5 | .2 | tr. | .6 | .3 | .3 | .7 |
| 11-Hydroxyetiocholanolone | 1.3 | .4 | .1 | — | .2 | .5 | .4 | — |
| 11-Hydroxyandrosterone | .2 | .6 | .1 | .3 | .4 | .5 | .3 | .8 |
| Etiocholanolone | .5 | — | .4 | .3 | .6 | .6 | .5 | 2.6 |
| Androsterone | .2 | — | .3 | tr. | .3 | .4 | .2 | 1.5 |
| Hydrolysis | A | D | B | B | C | D | | |
| Sex | M | F | M | M | M | M | | |
| Patient-age | M45 | H55 | B58 | S59 | H62 | B68 | | |

TABLE II

*Subject R29—The qualitative and quantitative steroid response to ACTH in a case of acute myelogenous leukemia*

| Compounds | Periods I ACTH 100 mg./day 9 days | II ACTH 100 mg./day 9 days | III Control 6 days | IV Control 6 days | V ACTH 100 mg./day 9 days | VI ACTH 200 mg./day 9 days |
|---|---|---|---|---|---|---|
| | *Milligrams per 24 hours* | | | | | |
| $C_{19}$ 11-oxysteroids | 3.0 | 4.0 | .7 | .2 | 1.1 | 4.2 |
| $C_{19}$ 11-desoxysteroids | 6.4 | 14.2 | 4.2 | 3.3 | 4.4 | 15.1 |
| 17α-Hydroxypregnanolone | 2.3 | 1.7 | — | .5 | 1.3 | 6.7 |
| 11-Ketoetiocholanolone | .9 | 1.4 | .4 | .2 | .3 | .8 |
| 11-Hydroxyetiocholanolone | .9 | 1.4 | — | — | tr. | tr. |
| 11-Hydroxyandrosterone | 1.2 | 1.2 | .3 | tr. | .8 | 3.4 |
| Etiocholanolone | 5.1 | 11.5 | 3.0 | 1.4 | 2.4 | 11.4 |
| Androsterone | 1.3 | 2.7 | 1.2 | 1.9 | 2.0 | 3.7 |
| Hydrolysis | Method C | | | | | |
| Sex | Male | | | | | |
| Patient-age | R 29 | | | | | |

the lower limit of the normal range for men in this age group. The level of $C_{19}$ 11-desoxysteroids was also at the lowest level observed for normal men.

*Subject B68:* This subject was a white man, admitted to the hospital six months previous to study, with a one-month history of adenopathy and a reportedly high white blood cell count. The diagnosis of chronic lymphatic leukemia was established by marrow biopsy. Five months previous to study, this subject had received $P^{32}$ therapy with a remission of his disease. His adenopathy and hepato-splenomegaly had recurred, however, at the time of study. He appeared well, otherwise.

The urinary ketosteroid values were lower than average although no striking alterations are evident.

*Acute myelogenous leukemia*

*Subject R29:* This patient was a white male physician, admitted to the hospital one day previous to study, with a one-month history of muscle and bone pain, and with an abnormally high white blood cell count. The diagnosis of acute myelogenous leukemia was established by marrow aspiration. He was acutely ill and it was impossible to obtain a control period preceding his first course of ACTH therapy. During this first therapeutic period he received 100 mg. per day of ACTH intramuscularly for a total of 24 days. Urinary steroids were studied during the first nine days (Period I) and the last nine days (Period II) of therapy. There was a striking clinical, metabolic and hematologic response to this treatment (24). Twelve days after the withdrawal of ACTH therapy, and during the period of clinical and hematologic remission, a control period (Period III) was obtained and the subject was discharged. Ten days later he was re-admitted because of increasing weakness and adenopathy. Marrow aspiration at this time showed complete relapse of his disease. A six-day control study (Period IV) was obtained following which he was again treated with 100 mg. per day of ACTH intramuscularly for nine days (Period V). During this period, edema of the face developed, although no eosinophile response was evident. Hematologic relapse persisted. The dose of ACTH was increased to 200 mg. per day at the end of this nine-day period. During the succeeding six days, there was an eosinophile response, but no hematologic remission of his disease. Period VI records the urinary steroid levels during the last eight days of ACTH therapy. He received 200 mg. per day of ACTH during this period, except for the last 48 hours during which the dose was increased to 400 mg. per day. Balance studies during this course of ACTH revealed a significantly smaller catabolic response than that observed during the first course of ACTH therapy. Bleeding persisted throughout this period and no hematologic response was evident. He expired in shock from profuse hemorrhage during the last day of this period, 23 days after the beginning of this second and unsuccessful trial of ACTH therapy.

The urinary ketosteroid data obtained during the periods studied are shown in Table II. The control data (Period III) for the first course of ACTH were obtained during a period beginning 12 days after the last dose of adrenocorticotropic hormone. It has already been established that the return to normal levels of urinary ketosteroids occurs within this period (26).

The levels of 11-oxygenated and 11-desoxysteroids during this control period were well below the average found in men in this age group. In this respect, the ketosteroid pattern of this subject is analogous to those of the older subjects described above. There is good evidence of adrenal response during ACTH administration from the clinical, hematologic, and metabolic observations during these periods. This is confirmed by the urinary ketosteroid findings, although it might be noted that the response in 11-oxygenated $C_{19}$ metabolites is smaller than that observed previously in normal men receiving similar treatment for experimental purposes (27).

The second control period (Period IV) followed 10 days upon the first, and was obtained during a period of acute relapse in his disease. Some diminution in ketosteroid excretion is evident. The adrenal response to ACTH during the first nine days of this second course of therapy was smaller than that observed during the first course. The response to intense stimulation with ACTH during the last eight days of therapy was evident. Individual differences in 11-oxygenated steroid levels were noted during this period, as compared with the similar period of the first course of therapy.

## DISCUSSION

There appear to be alterations in urinary ketosteroids in the seven subjects studied. In all patients the $C_{19}$ 11-desoxysteroid metabolites were well below the average for the appropriate age and sex. It was not possible, in fact, to identify androsterone and etiocholanolone in the urine of Subject H55. While Subject R29 excreted more of these steroids than the other patients, comparison with a series of normal men in this age group (22) suggests a relative depression of these values. A simliar depression of the 11-oxygenated metabolites was observed in most subjects. Thus, subject R29, S59, H62, and B68 all showed low levels of 11-oxygenated steroid metabolites.[2] A comparison of the average ketosteroid values for the male leukemic subjects, 45 years or older, with those of normal men of similar age, is presented in Table I. A pronounced difference in steroidal excretion exists between these two groups.

While the experimental findings show clearly that the steroid excretion is low in the leukemic subjects, the reasons for this are less clearly evident. From the data, the alterations in steroid excretion may precede or result from physiologic changes induced by the subject's disease or they may be the result of a chronic, debilitating illness. In addition many other credible or specious explanations could be advanced for the results observed. These might include changes in the rate of hormone metabolism, increased "utilization" of hormones or metabolites, alteration in renal clearance, production of unusual, non-chromogenic steroids, intestinal rather than urinary excretion of metabolites, formation of chemically stable, unhydrolyzed conjugates, increased tissue storage, and the like. Regardless of the inherent merit of these possibilities, it is clear from the results reported that at least one of the subjects exhibited a clear response to ACTH by an increased excretion of urinary steroid hormone metabolites. In this subject, then, it has been demonstrated that his metabolic functions with respect to the adrenal steroid hormones were normally operative and qualitatively identical with that of other subjects (27). Furthermore, it has been demonstrated that a patient with chronic lymphatic leukemia (Subject F1) handled a tracer dose of hydrocortisone-4-$C^{14}$ in a manner identical with other patients not afflicted with this disease (28). It seems, therefore, unreasonable to invoke remote possibilities when the consistently decreased excretion of steroid metabolites can reasonably be ascribed to a decrease in the output of the steroid producing glands. Therefore, the interpretation that the urinary steroids reflect the hormonal se-

[2] Relative to this group of metabolites, the application of more precise methods of hydrolysis and separation techniques results in the identification of 3$\alpha$,11$\beta$-dihydroxyetiocholane-17-one, in the urine of most normal subjects, contrary to previous observations (22). Similarly, small amounts of 3$\alpha$,17$\alpha$-dihydroxypregnane-20-one are normally identified by these techniques.

cretion of the adrenal and gonads is preferred. It should be emphasized that this is a rational explanation of the facts developed by the methods available when these studies were made and not a rigorous demonstration of proven fact.

With these considerations in mind, it is of interest to consider the studies in subject R29 during the two courses of ACTH therapy. The urinary ketosteroids of R29 were at low levels during the first control period. In other respects the ketosteroid pattern was not unusual. The response to ACTH during the first course of therapy was similar to that observed in normal subjects, although the response in 11-oxygenated metabolites appeared somewhat diminished. Nevertheless, during these ACTH periods, pronounced metabolic, clinical, and hematologic responses were obtained (24). The control period obtained immediately after the acute relapse of his disease shows a steroid pattern different from that obtained during a period of complete remission. In view of the established reproducibility of the ketosteroid pattern in man (26), the steroid changes observed in this subject may be related to the change in his clinical status.

The observed changes in levels of 11-oxygenated (adrenal origin) and 11-desoxy (adrenal and gonadal origin) steroids while not pronounced, are suggestive of decreased adrenocortical activity. The somewhat diminished steroid response to ACTH during the first nine days of the second course of therapy and the diminished catabolic response observed during this same period were perhaps reflections of this alteration. Here again, however, the nature and severity of the illness as well as the kind and route of ACTH administration in this subject cannot be discounted. The question of whether the quantitative steroid and metabolic responses to ACTH are reproducible in all respects limits speculation from these data. It does appear, however, that the physiologic events suggested by the steroidal changes, and the abrupt change in clinical status may be of more than coincidental significance.

## SUMMARY

The relationship between gonadal and adrenocortical function and human leukemia has been investigated by means of detailed isolation studies of the urinary ketosteroids in seven patients; six with chronic lymphatic leukemia and one with acute myelogenous leukemia during therapy with adrenocorticotropic hormone. Evidence of diminished gonadal and/or adrenocortical secretory activity in these patients was obtained. In one patient, clinical relapse and the development of refractoriness of the leukemia to ACTH therapy seemed related to disordered adrenal function, as determined by the qualitative and quantitative steroid response to ACTH.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of grants from the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council), the Anna Fuller Fund, the Lillia Babbitt Hyde Foundation, and the National Cancer Institute of the National Institutes of Health of the United States Public Health Service, (C-322).

The authors wish to express their deep appreciation to Dr. Olaf Pearson of the Division of Clinical Investigation, Sloan-Kettering Institute, for his generous cooperation in these studies. The authors also wish to express their appreciation to the large group of devoted Research Assistants and technicians who made much of the work possible. The routine chemical and chromatographic separations were carried out by a group under the supervision of Madeleine Stokem and Ruth Jandorek. The colorimetric analyses were under the supervision of Olga Teager and Denise O'Hara. The infrared spectrometry was under the supervision of Friederike Herling.

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# STUDIES ON COPPER METABOLISM. XIII. HEPATOLENTICULAR DEGENERATION [1]

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(Submitted for publication May 18, 1954; accepted July 6, 1954)

It has now been demonstrated repeatedly that in patients with hepatolenticular degeneration there is a disturbance in the metabolism of copper and amino acids. The amount of copper in the tissues, particularly in the liver and involved areas of the brain, is increased about ten-fold above the normal (1–4). The pigmented Kayser-Fleischer rings have been shown to contain copper (5, 6). Increased excretion of copper in the urine has also been demonstrated (7–9). The plasma copper level was originally considered to be normal or increased (3, 9, 10), but recent studies have shown that the plasma copper level is decreased (11–13). Normally, about 96 per cent of the copper in plasma is bound to a specific alpha-2 globulin, ceruloplasmin (14, 15). The remainder of the copper in plasma is more loosely bound to protein and is capable of reacting directly with sodium diethyldithiocarbamate (15). This direct-reacting fraction actively transports copper (15). It has now been demonstrated that in patients with Wilson's disease there is a reduction in the ceruloplasmin fraction (16, 17). Measurements of the direct-reacting fraction have not been made, but it has been noted that the ceruloplasmin content of the serum is consistently too low to account for the copper present (13, 16).

The aminoaciduria which accompanies this disease (18) involves most of the amino acids found in normal urine, but, in addition, proline and citrulline are excreted (19, 20). The urinary amino acid pattern varies widely, depending upon the stage of the disease and the protein composition of the diet. The aminoaciduria is independent of the severity of the hepatic disorder. It occurs even in patients with no manifest liver disease (21). No significant elevation of the blood alpha amino nitrogen level has been observed (10, 20, 22). It has been suggested that the aminoaciduria is the result of a renal lesion (20).

In addition to the aminoaciduria, an increased excretion in the urine of oligopeptides with dicarboxylic acids as terminal residues has been observed (21). Evidence has been presented that the copper is excreted in the urine, at least in part, in the form of an oligopeptide-copper chelate (23).

Various workers have demonstrated that following the administration of B.A.L. (dimercaptopropanol) (6, 7, 9, 10), glycine (10), alanine (10), "Versene" (calcium disodium salt of ethylenediamine tetra-acetic acid) (13), a high protein diet (13), and cortisone (13), the excretion of copper in the urine is enhanced. Clinical improvement has been reported in some patients following repeated courses of B.A.L. (6).

At the present time there is no agreement as to which of the metabolic defects is inherited and primary. Uzman (23) has proposed that the aminoaciduria is the primary defect since he observed this abnormality to be the single most constant metabolic feature of the disease, present even in five asymptomatic siblings of affected patients (21). However, Bearn and Kunkel (13) point out that the family studied by Uzman and Hood (21) is genetically atypical because of the large number of biochemically affected individuals described. A study of 35 siblings of affected patients failed to reveal aminoaciduria in any individual. Furthermore, increased amino acid excretion was absent in one patient. Bearn and Kunkel (13) conclude that aminoaciduria is not the primary defect or earliest manifestation of this disease and suggest that the fundamental defect is an increased absorption of copper. Scheinberg and Gitlin (16) have proposed that the inherited defect is a deficiency of ceruloplasmin. As yet

[1] This investigation was supported by a research grant (RG 25) from the National Institutes of Health, United States Public Health Service.

abnormalities in copper metabolism have not been sought adequately in asymptomatic siblings.

Two different concepts concerning the metabolic factors operative in the pathogenesis of hepatolenticular degeneration have been proposed. Uzman (23) and Brick (24) suggested that the development of cirrhosis of the liver is a result of the chronic loss of amino acids. They call attention to the similarity of the de Toni-Fanconi syndrome with the same combination of aminoaciduria and cirrhosis. These investigators attempt to explain the accumulation of copper in the tissues as the result of a chelation between the copper and peptide residues, the latter accumulating in the tissues as a consequence of a fault in the metabolism of dicarboxylic amino acid peptides. They suggest, furthermore, that the oligopeptide-copper chelates pass from the tissues to the plasma and into the glomerular filtrate. The increased urinary excretion of copper would then be the result of the competition for tubular reabsorption between the oligopeptide complex and the amino acids present in high concentration in the tubular fluid.

On the other hand, Bearn and Kunkel (13) suggest that, as a result of increased absorption, copper accumulates in the tissues and, as a consequence of excessive deposition of copper in the liver and kidney, cirrhosis and damage to the tubular epithelium occur. This would be somewhat analogous to the development of cirrhosis of the liver secondary to the deposition of excessive amounts of iron in the liver, such as occurs in patients with hemochromatosis. The increased excretion of amino acids, peptides, and occasionally glucose would then be the result of the damage to the tubular epithelium, and the increased excretion of copper in the urine is interpreted as the consequence of the high tissue level of copper and the aminoaciduria and peptiduria.

The purpose of this communication is to report studies undertaken to explore further the abnormalities in copper metabolism in patients with hepatolenticular degeneration. On the basis of these studies a modified concept of the pathogenesis of the disease will be presented.

## METHODS

The methods for the determination of total plasma copper (25), the direct-reacting fraction of plasma copper (15), erythrocyte copper (25), tissue copper (26), urine copper (27), plasma iron (28), the total iron-binding capacity of the plasma (29), urine protein (27), and electrophoretic analysis of the plasma proteins (30) have been described in previous publications. Total plasma protein, albumin, and globulin were determined by the biuret method (31) with the modification of Weichselbaum (32). Urinary alpha amino nitrogen was determined by the gasometric ninhydrin method of Van Slyke, MacFadyen, and Hamilton (33).

During the period of the copper balance studies the patients were hospitalized on a metabolic ward. The exact amount of each foodstuff eaten by each patient was weighed daily. An equal amount of each foodstuff was placed in a suitable container, sulfuric acid was added, and the material was digested slowly over a hotplate. The digests were then combined for each day and homogenized in a Waring blendor. The total volume was recorded and the amount of copper in an aliquot was determined. Total 24-hour urine collections were made daily. All stools were collected for the period, combined and processed as noted above for the food. The water intake was recorded daily and the amount of copper in the water was determined but was found to be insignificant (0.2 $\mu$g per 100 ml.). Particular care was exercised to avoid contamination of the specimens by copper. All glassware used was carefully cleaned with acid, rinsed with copper-free water, and air-dried. The amount of copper in the stool and food digests was determined by the method used for the determination of copper in urine.

## RESULTS

### *Clinical data*

Seven patients with hepatolenticular degeneration have been studied in detail. Total plasma copper and red cell copper were determined in one additional patient.[2] All seven patients had well developed Kayser-Fleischer corneal rings. With one exception (Dar. H.) to be discussed later, all of the patients manifested signs of neurologic disease such as tremor, dysarthria, and mental disturbances. Rigidity of the skeletal muscles was moderate to advanced in four of the patients. All patients except one (Del. H.) are still living. Additional clinical details are given in Table I. Liver function studies, plasma iron, and the total iron-binding capacity of the plasma are presented in Table II. No patient had glycosuria.

Family H. is of particular interest. The parents are fourth cousins and have had five children. The oldest child (Del. H.) is described in detail

[2] We are indebted to Dr. Ian Brown, Department of Neurology, University of Minnesota, for sending us a specimen of blood from this patient.

TABLE I

*Clinical data*

| Patient | Age yrs. | Age at onset yrs. | Sex | Parental consanguinity | Siblings No. | Siblings No. involved | Type of onset | Hepatomegaly | Splenomegaly |
|---|---|---|---|---|---|---|---|---|---|
| D. C. | 28 | 25 | F | None | 8 | 0 | Tremor of arms | + | + |
| Del. H. | 15 | 9 | M | 4th Cousin | 4 | 2 | Dysarthria | 0 | + |
| Dar. H. | 10 | 8 | F | 4th Cousin | 4 | 2 | Hemolytic anemia | + | + |
| R. K. | 21 | 9 | M | None | 1 | 0 | Purpura | + | +* |
| W. K. | 30 | 26 | M | None | 2 | 0 | Tremor of arms | + | + |
| M. L. | 20 | 18 | M | None | 5 | 0 | Dysarthria | 0 | 0 |
| J. S. | 29 | 26 | F | None | 2 | 0 | Dysarthria | 0 | 0 |

* Splenectomy for purpura at age 9.

in this paper. The next oldest child (La. H.), at the age of six, suddenly developed jaundice which was accompanied by vomiting, fever, anemia, hepatomegaly, and ascites. The degree of jaundice deepened progressively and the child died six weeks later. She had apparently been quite well prior to the onset of this illness and neither before or during the illness were any neurological abnormalities noted. The patient was not under our observation at any time and died in another hospital. Autopsy revealed cirrhosis of the liver. Examination of the brain was not performed and the eyes were not examined for the presence of Kayser-Fleischer rings.

The third child (Dar. H.) was examined after the diagnosis of Wilson's disease was made in the oldest sibling (Del. H.). Physical examination revealed a healthy looking, well-nourished, alert girl nine years of age. Spider angiomata were observed over the shoulders. The liver and the spleen were both palpable 2 cm. below the costal margin. The remainder of the physical examination was not unusual. No neurologic abnormalities were present. Past history revealed that at age eight the patient had an illness characterized by nausea, vomiting, fever, and jaundice. Laboratory examinations performed in another hospital revealed a hemoglobin of 4.4 gm. per 100 ml.; reticulocytes, 24 per cent; leukocyte count, 22,800 per mm.$^3$; total serum bilirubin, 7 mg. per 100 ml. Examination of the blood smear showed anisocytosis, a marked degree of polychromatophilia and a few nucleated red blood cells. The patient was given several transfusions of whole blood and was thought to have recovered rapidly and completely. Since this illness she has remained asymptomatic and active.

Examination of the fourth child (Le. H.), age 5, and the fifth child (C. H.), age 1, has failed to reveal any significant abnormalities on physical examination. The plasma copper levels were 155 and 169 $\mu$g per 100 ml., respectively. The urinary levels of copper and alpha amino nitrogen were within the normal range and liver function studies were not remarkable.

Examination of both parents and the four grandparents has also failed to reveal any signifi-

TABLE II

*Liver function studies, plasma iron, and total iron-binding capacity of the plasma*

| Patient | Total plasma proteins gm./100 ml. | Plasma albumin gm./100 ml. | Plasma globulin gm./100 ml. | B.S.P.* % retention | Thymol turbidity units | Ceph. floc. | Total plasma bilirubin mg./100 ml. | Plasma iron† µg/100 ml. | T.I.B.C.‡ µg/100 ml. |
|---|---|---|---|---|---|---|---|---|---|
| D. C. | 6.0 | 3.2 | 2.8 | 32 | 6 | 2† | 2.2 | 97 | 187 |
| Del. H. | 4.8 | 1.0 | 3.8 | 28 | 12 | 4† | 2.8 | 23 | 101 |
| Dar. H. | 5.7 | 2.9 | 2.8 | 26 | 7 | 0 | 1.6 | 82 | 217 |
| R. K. | 6.7 | 3.7 | 3.0 | 8 | 5 | 0 | 0.6 | 24 | 184 |
| W. K. | 7.4 | 3.8 | 3.6 | 5 | 7 | 4† | 0.5 | 70 | 180 |
| M. L. | 6.2 | 4.1 | 2.1 | 11 | 4 | 1† | 0.8 | 71 | 221 |
| J. S. | 6.5 | 3.3 | 3.2 | 2 | 5 | 0 | 0.6 | 122 | 172 |

* Forty-five minutes after 5 mg. per kg. of body weight.
† Normal value, 110 ± 31 µg per 100 ml.
‡ T.I.B.C., total iron-binding capacity of the plasma. Normal value, 359 ± 30.8 µg per 100 ml.

cant abnormalities either by physical examination or by laboratory studies.

*Plasma copper*

The total plasma copper levels were significantly reduced in all eight patients (Table III). The lowest plasma copper level which we have observed in a study of a total of 228 normal subjects is 68 μg per 100 ml. The values in all eight patients were below this lowest normal value. The mean plasma copper value for the patients with Wilson's disease was 50 μg per 100 ml. as compared with a mean normal value of 116 μg per 100 ml.

The absolute amount of direct-reacting copper in the plasma of the patients was significantly increased (Table III) above the normal. The mean value for the group was 26 μg per 100 ml. as compared with a mean value of 8 μg per 100 ml. in the normal control group. In only one of the patients was the value within the normal range. In the normal subjects, 7 per cent (range, 0 to 23) of the total plasma copper was direct-reacting. In the patients with hepatolenticular degeneration, 52 per cent (range, 42 to 68) of the total plasma copper was in the direct-reacting fraction.

Subtraction of the direct-reacting fraction from the total plasma copper (Table III) results in a mean value of 24 μg per 100 ml. for the indirect-reacting fraction in the patients, as compared with a mean value of 108 μg per 100 ml. for the indirect-reacting fraction in normal subjects.

*Spinal fluid copper*

The concentration of copper in the spinal fluid was determined in six of the patients. The results are presented in Table III and are compared with the values obtained in 11 patients with various forms of neurologic disease or suspected neurologic disease other than hepatolenticular degeneration. The control group cannot be considered as normal, since in many of the patients studied the plasma copper level was elevated. In spite of this, in all six of the patients with Wilson's disease, the concentration of copper in the spinal fluid was increased above the control range and the mean value was three times the mean value observed in the control patients. Determination of the amount of spinal fluid copper reacting directly with sodium diethyl-dithiocarbamate revealed that in all six fluids obtained from the patients with Wilson's disease, all of the copper reacted directly. In the control group only about one-half of the spinal fluid copper reacted directly.

*Erythrocyte copper*

The amount of copper in the erythrocytes was determined in eight of the patients (Table III). The mean value (129 μg per 100 ml.) was not significantly different from the normal mean (115 μg per 100 ml.). In one patient (R. K.), the red cell copper was increased above the normal range.

TABLE III

*Plasma, spinal fluid, erythrocyte and urine copper and urine alpha amino nitrogen and protein*

| Patient | | (A) Total plasma copper μg/100 ml. | (B) Direct-reacting plasma copper μg/100 ml. | A − B μg/100 ml. | Spinal fluid copper μg/100 ml. | R.B.C. copper μg/100 ml. | Urine copper μg/day | Urine α-amino nitrogen mg./day | Urine protein gm./day |
|---|---|---|---|---|---|---|---|---|---|
| Normal | No. | 228 | 19 | 19 | 11 | 31 | 10 | 15* | 10 |
| | Mean | 116 | 8 | 108 | 6 | 115 | 9 | 164 | 0.05 |
| | ±Stand. dev. | 14 | 6.8 | | 2.3 | 22 | 8.1 | | 0.02 |
| | Range | 68–161 | 0–20 | 66–150 | 3–9 | 84–159 | 0–26 | 118–204 | 0.02–0.08 |
| D. C. | | 65 | 27 | 38 | 10 | 97 | 415 | 397 | 0.22 |
| Del. H. | | 33 | 12 | 21 | 16 | 130 | 115 | 295 | 0.30 |
| Dar. H. | | 40 | 27 | 13 | | 111 | 122 | 90 | 0.16 |
| R. K. | | 44 | 23 | 21 | 18 | 212 | 119 | 484 | 0.33 |
| W. K. | | 60 | 31 | 29 | 16 | 103 | 493 | 519 | 0.17 |
| M. L. | | 47 | 22 | 25 | 24 | 138 | 611 | 271 | 0.34 |
| J. S. | | 60 | 41 | 19 | 24 | 112 | 236 | 303 | 0.12 |
| J. T. | | 52 | | | | 130 | | | |
| Mean | | 50 | 26 | 24 | 18 | 129 | 302 | 337 | 0.23 |

* Values given by Cooper, Eckhardt, Faloon, and Davidson (22).

*Urine copper*

Representative values for the daily excretion of copper in the urine are given in Table III. In all of the patients, the urinary excretion of copper was increased well beyond the normal range of 0 to 25 μg per 24 hours. The amount of copper excreted per day by a given patient varied somewhat from one period to another but rarely was it less than 100 μg per day. The highest single value observed in any patient without therapy was approximately 850 μg per 24 hours. In general, the excretion of copper in the urine ranged from 4 to 30 times the maximal normal value.

It should be noted that during the periods of study no attempt was made to maintain a constant intake of protein. Bearn and Kunkel (13) have shown that with an increase in dietary protein, there is an increase in the excretion of amino acids and copper in the urine.

It has been demonstrated in this laboratory (27) that in patients with the nephrotic syndrome, the amount of copper excreted in the urine is directly proportional to the amount of protein in the urine. In such individuals approximately 31 μg of copper were excreted per gram of protein. As shown in Table III, in patients with hepatolenticular degeneration, only traces of protein were excreted in spite of pronounced hypercupriuria.

*Urine alpha amino nitrogen*

The urinary excretion of alpha amino nitrogen was increased in six of the seven patients studied (Table III). In the seventh patient (Dar. H.) the values of 73 and 107 mg. per 24 hours (1.12 and 1.64 mg. per lb. of body weight per 24 hours) were within the normal range of 0.71 to 1.98 mg. per lb. of body weight per day given for children (34).

The values recorded in Table III represent the means of several determinations. Measurements of the urinary copper were not made on the same specimens as those in which amino acid was measured. When alpha amino nitrogen and copper determinations were performed on the same sample of urine, there was good correlation (correlation coefficient, + 0.78) between the amounts of alpha amino nitrogen and copper. This is similar to the finding of Bearn and Kunkel (13). It should be noted, however, that in the patient (Dar. H.) who excreted normal amounts of alpha amino nitrogen there was a significant degree of hypercupriuria.

*Electrophoretic analysis of plasma proteins*

Electrophoretic analyses were performed on the sera of two patients and on the plasma of five patients (Table IV). In several of the patients there was a reduction in the albumin fraction and an increase in the gamma globulin fraction. These changes were consistent with the degree of liver damage in these patients. The amount of alpha globulin was not reduced. This indicates that in such patients there is no over-all inability to maintain the alpha globulin level even though the alpha-2 globulin, ceruloplasmin, is greatly decreased.

*Tissue copper*

Analyses of the tissues for copper were made on the one patient (Del. H.) who died during the course of the study. The results are presented in Table V and are compared with those obtained on a 30-year old male subject, weighing 250 pounds, who was killed in an automobile accident. The

TABLE IV

*Electrophoretic analyses of the plasma proteins*

| Patient | Total protein gm./100 ml. | Albumin gm./100 ml. | $\alpha_1$ gm./100 ml. | $\alpha_2$ gm./100 ml. | $\alpha_3$ gm./100 ml. | $\beta_1$ gm./100 ml. | $\beta_2+\beta_3$ gm./100 ml. | $\gamma$ gm./100 ml. | $\phi$ gm./100 ml. |
|---|---|---|---|---|---|---|---|---|---|
| D. C.* | 5.80 | 2.62 | 0.35 | 0.47 | | 0.40 | 0.34 | 1.62 | |
| Del. H.* | 6.22 | 1.87 | 0.62 | 0.43 | 0.37 | 0.50 | 0.43 | 1.99 | |
| Dar. H. | 5.90 | 3.02 | 0.41 | 0.41 | 0.06 | 0.53 | 0.18 | 1.00 | 0.29 |
| R. K. | 7.27 | 3.71 | 0.29 | 0.46 | | 1.20 | | 0.87 | 0.74 |
| W. K. | 8.12 | 4.40 | 0.41 | 0.85 | | 1.00 | | 1.00 | 0.46 |
| M. L. | 7.35 | 4.38 | 0.52 | 0.96 | | 0.71 | | 0.65 | 0.13 |
| J. S. | 6.22 | 3.18 | 0.55 | 0.73 | | 0.69 | | 0.67 | 0.40 |

* Serum.

TABLE V

*Tissue copper analyses on a normal control subject and on a patient (Del. H.) with hepatolenticular degeneration*

| Tissue | Normal subject | | | Del. H. | | |
|---|---|---|---|---|---|---|
| | Dry wt. μg/gm. | Wet wt. μg/gm. | Total organ μg | Dry wt. μg/gm. | Wet wt. μg/gm. | Total organ μg |
| Liver | 12.2 | 3.9 | 8,112 | 532.0 | 98.4 | 49,200 |
| Spleen | 3.3 | 0.7 | 126 | 7 7 | 1.5 | 300 |
| Kidney (R) | 10.0 | 1.9 | 256 | 47.2 | 8.7 | 2,310 |
| Adrenal gland (R) | 5.0 | 1.5 | 8 | 9.6 | 2.6 | 30 |
| Heart | 12.2 | 3.8 | 1,178 | | 3.0 | 510 |
| Lung (R) | 6.7 | 1.2 | 330 | 15.2 | 1.6 | 320 |
| Muscle | 4.6 | 1.0 | | 10.0 | 1.9 | |
| Pancreas | | | | 4.2 | 1.7 | |
| Bile (*per 100 ml.*) | | 24.0 | | | 19.0 | |
| Spinal cord | 12.0 | 3.0 | | 109.0 | 27.3 | |
| Cortex (white matter) | 13.9 | 4.3 | | 144.0 | 41.7 | |
| Cortex (gray matter) | 15.9 | 3.8 | | 327.0 | 65.3 | |
| Cerebellum (white matter) | 23.3 | 5.3 | | 247.0 | 74.2 | |
| Cerebellum (gray matter) | 33.0 | 7.0 | | 337.0 | 67.4 | |
| Basal ganglion (R) | 24.6 | 6.0 | | 225.0 | 56.4 | |
| Brain stem | 8.0 | 2.0 | | 237.0 | 58.6 | |

results are not entirely comparable since Del. H. was 15 years of age and weighed 80 pounds.

In the patient with Wilson's disease, as compared with the normal subject, the concentration of copper was increased in all of the tissues except the heart muscle. The increase was most marked in the liver and brain stem. However, the concentration of copper (wet weight) was increased 9 to 17 times that of the control value in all areas of the nervous system which were studied, including the spinal cord. The concentration of copper in the lung and in muscle was increased only slightly.

*Copper balance studies*

Copper balance studies were carried out in four patients with Wilson's disease and in three normal control subjects. Because it has been demonstrated that in patients with infection or with active rheumatoid arthritis there is an increase in the plasma copper level (35) and that under these circumstances there is an increased retention of copper in the body (36), balance studies were also carried out for comparison in two patients with active rheumatoid arthritis.

The results are presented in Table VI. Because the average daily copper intake was not the same in all individuals, the differences between intake and output are expressed in proportion to each mg. of copper ingested as well as in absolute amounts. It must be emphasized that, because of difficulties in analysis and collections, copper balance studies are extremely difficult to carry out accurately and the values given in the table represent only approximations.

In the normal control subjects, intake and output of copper were essentially the same (+ 0.01 mg. of copper per day per mg. of copper intake). The patients with hepatolenticular degeneration were in positive copper balance to the extent of about 0.56 mg. of copper per day per mg. of copper ingested. The patients with active rheumatoid arthritis were also in positive copper balance (0.27 mg. of copper per day per mg. of copper ingested), although less so than the patients with Wilson's disease.

*The influence of potassium sulfide and of casein hydrolysate on copper balance*

In order to determine whether a state of negative copper balance could be produced in patients with Wilson's disease, one of the patients (J. S.), after a control period of 10 days was given 20 mg. of potassium sulfide [3] by mouth three times daily with meals for a total of ten days. The rationale of giving potassium sulfide orally was that insoluble copper sulfide would be formed in the gastro-intestinal tract. It has been demonstrated in animals that copper sulfide is not absorbed (37). The period of potassium sulfide administration was then followed by a second control period of 11 days, and then one liter of casein hydrolysate was given slowly by the intravenous

[3] Potash sulfurated technical (Maelinckrodt).

TABLE VI

*Copper balance studies*

| Group | Normal controls | | | Rheumatoid arthritis | | Hepatolenticular degeneration | | | |
|---|---|---|---|---|---|---|---|---|---|
| Subject | A. G. | T. C. | B. H. | H. S. | V. H. | J. S. | W. K. | R. K. | M. L. |
| Period, *days* | 10 | 10 | 10 | 7 | 7 | 10 | 11 | 11 | 12 |
| Intake, *mg./day* | 2.62 | 2.62 | 2.62 | 1.87 | 2.57 | 1.38 | 3.82 | 3.79 | 2.44 |
| Output: | | | | | | | | | |
| Urine, *mg./day* | 0.04 | 0.01 | 0.04 | 0 | 0 | 0.26 | 0.40 | 0.11 | 0.15 |
| Stool, *mg./day* | 2.99 | 2.55 | 2.21 | 1.41 | 1.79 | 0.53 | 1.30 | 0.96 | 1.02 |
| Total, *mg./day* | 3.03 | 2.56 | 2.25 | 1.41 | 1.79 | 0.79 | 1.70 | 1.07 | 1.17 |
| Intake minus output *mg./day* | −0.41 | +0.06 | +0.37 | +0.46 | +0.78 | +0.59 | +2.12 | +2.72 | +1.27 |
| Intake minus output *mg./day/1 mg. Intake* | −0.15 | +0.02 | +0.14 | +0.25 | +0.30 | +0.43 | +0.56 | +0.72 | +0.52 |
| Mean for each group *mg./day/1 mg. Intake* | | +0.01 | | | +0.27 | | | +0.56 | |

route daily for eight days in order to increase the excretion of copper through the kidneys.

The results are presented in Figure 1. The administration of potassium sulfide was associated with an increased excretion of copper in the stools but not in the urine, with the result that the overall copper balance changed from + 0.43 to − 0.14 mg. per day per mg. of copper intake. During the second control period, positive copper balance was re-established. The administration of casein hydrolysate was associated with an increased excretion of copper in both the urine and feces with the result that the copper balance was again changed from positive (0.21 mg. per day per mg. of copper intake) to negative (− 0.75 mg. per day per mg. of copper intake).

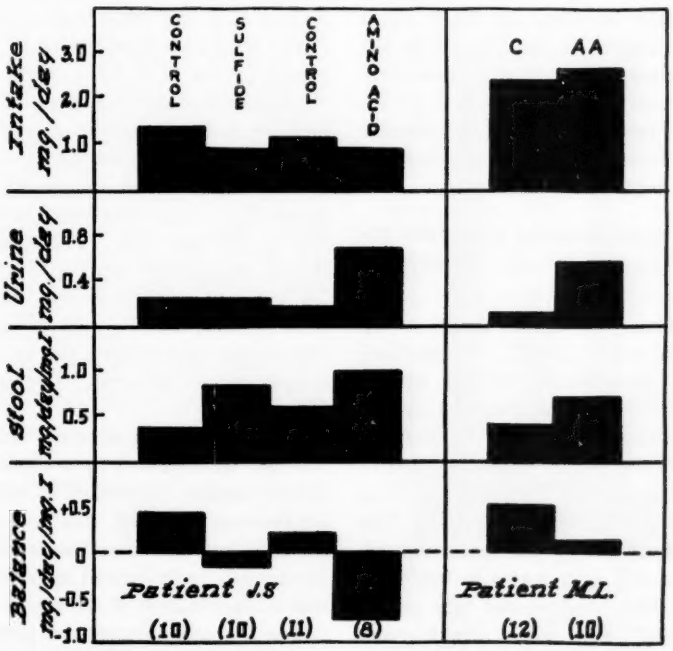


FIG. 1. THE INFLUENCE OF POTASSIUM SULFIDE (20 MG., THREE TIMES DAILY), AND CASEIN HYDROLYSATE (AMINO ACIDS, 50 GRAMS DAILY) ON COPPER BALANCE

TABLE VII

*The influence of various agents on the excretion of copper in the urine*

| Agent | Patient | Period: Control μg/day | Period: Therapy μg/day | Increase μg/day | % Increase |
|---|---|---|---|---|---|
| B.A.L. | D. C. | 423 | 974 | 551 | 130 |
| | D. C. | 287 | 1,237 | 950 | 330 |
| | D. C. | 241 | 1,085 | 844 | 350 |
| | Del. H. | 187 | 562 | 375 | 200 |
| | Del. H. | 220 | 563 | 343 | 156 |
| Casein hydrolysate | D. C. | 312 | 705 | 393 | 126 |
| | Del. H. | 101 | 538 | 437 | 432 |
| | M. L. | 152 | 565 | 413 | 272 |
| | J. S. | 180 | 693 | 513 | 286 |
| | R. K. | 106 | 381 | 275 | 260 |
| | W. K. | 442 | 907 | 465 | 105 |
| B.A.L. + Casein hydrolysate | D. C. | 256 | 1,111 | 855 | 367 |
| | Del. H. | 114 | 896 | 782 | 636 |
| I.V. "Versenate" | D. C. | 395 | 817 | 422 | 107 |
| | D. C. | 218 | 656 | 438 | 200 |
| | Del. H. | 161 | 217 | 56 | 35 |
| | W. K. | 362 | 785 | 423 | 117 |
| Oral "Versenate" | D. C. | 306 | 548 | 242 | 79 |
| | R. K. | 110 | 87 | 0 | 0 |
| Sulfide | J. S. | 267 | 273 | 6 | 4 |

A second patient (M. L.) was given one liter of casein hydrolysate intravenously daily for a 10-day period following a 12- day control period (Figure 1). Simultaneously with the administration of the amino acid mixture there was an increase in stool and urine copper and the over-all balance decreased from + 0.52 mg. per day per mg. of intake to + 0.13.

*The influence of various chelating agents and potassium sulfide on the urinary excretion of copper*

To study the influence of various agents on the urinary excretion of copper, B.A.L. (dimercaptopropanol), casein hydrolysate (amino acids), "Calcium Versenate" (calcium disodium salt of ethylene-diamine tetra-acetic acid), and potassium sulfide were administered to patients. B.A.L. was given intramuscularly in oil twice a day in an amount of 2.5 mg. per kg. of body weight. One liter of casein hydrolysate (5 per cent solution) was given intravenously over a two to four-hour period each day. Two hundred and fifty mg. of "Calcium Versenate" were given orally every hour for a total of 13 doses daily. "Calcium Versenate" was administered intravenously each day by adding two grams to 200 ml. of 5 per cent glucose. Potassium sulfide was given orally in an amount of 20 mg. three times a day with meals.

The results are summarized in Table VII. The administration of B.A.L., casein hydrolysate and "Calcium Versenate" was associated with a marked increase in the excretion of copper in the urine. These agents, in the amounts given, increased the urinary excretion of copper about 300 to 900 μg per day. This represented an increase of about 100 to 400 per cent. The mean increase with B.A.L. was 233 per cent; with casein hydrolysate, 247 per cent; with "Calcium Versenate," 115 per cent. When B.A.L. was given simultaneously with casein hydrolysate, the greatest increase in urinary copper occurred. The mean increase was 501 per cent.

The oral administration of "Calcium Versenate" was not accompanied by a significant increase in urinary copper. Although in one patient (D. C.) an increase occurred, this was probably due to the fact that "Calcium Versenate" was given intravenously in the period immediately prior to the oral administration of the compound (Figure 2).

The oral administration of potassium sulfide, as would be expected, was not associated with a rise in urinary copper (Table VII and Figure 1).

In Figures 2, 3, and 4, the influence of various

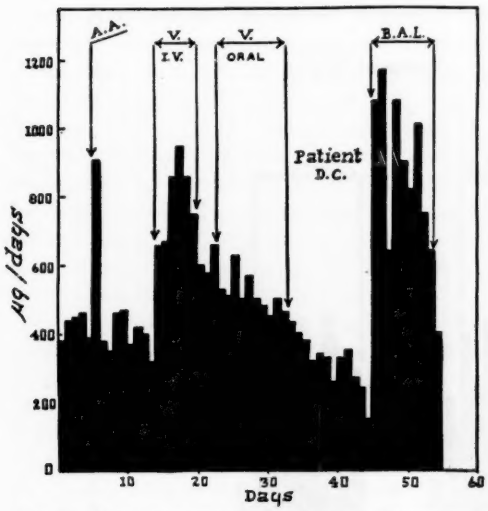


FIG. 2. THE INFLUENCE OF CASEIN HYDROLYSATE (AA), "CALCIUM VERSENATE" (V), AND B.A.L. (DIMERCAPTOPROPANOL) ON THE EXCRETION OF COPPER IN THE URINE

For experimental details consult the text.

agents on the excretion of copper in the urine is depicted graphically for purposes of comparison. It should be noted that there was no tendency for the amount of copper eliminated in the urine to decrease with each additional course of therapy. Likewise, after repeated courses, the baseline level of copper excreted was changed little or not at all. One patient (Del. H., Figure 4) was given B.A.L. for 15 consecutive days. In spite of the long course of B. A. L., and in spite of the fact that this followed soon after four other courses of therapy, there was little tendency for the amount of copper excreted to decrease.

Approximately 58 per cent of the copper excreted in the urine was dialyzable under the conditions stated in Table VIII. Following the intravenous administration of casein hydrolysate, the proportion of copper which was dialyzable remained the same, in spite of the fact that the total amount of copper excreted increased. Thus, following the administration of casein hydrolysate, there was also an increase in the non-dialyzable fraction of urinary copper. As would be expected, the per cent of dialyzable copper increased significantly following the administration of B.A.L. or "Versenate."

TABLE VIII

*Per cent of urine copper dialyzable* *

| Agent administered | Number of determinations | % Dialyzable |
|---|---|---|
| None | 10 | 58 (44–74) |
| Casein hydrolysate | 6 | 53 (41–67) |
| B.A.L. | 3 | 77 (72–81) |
| "Calcium Versenate," I.V. | 1 | 84 |

* Cellophane casing (Visking Corp.). Twenty ml. of urine were dialyzed against one liter of distilled water for 24 hours at 5° C. The water was changed four times during this period.

Five of the patients (Table IX) were treated with 20 mg. of potassium sulfide by mouth with meals three times daily for periods of four to 12 months. After such therapy, there was no significant change in the plasma copper level and the urinary excretion of copper was not significantly less. Four of the patients received no other form of therapy during this period of time. Clinical evaluation of the results was difficult. Although striking clinical improvement did not occur in any of the patients, it was our impression that the disease did not progress in any of them.

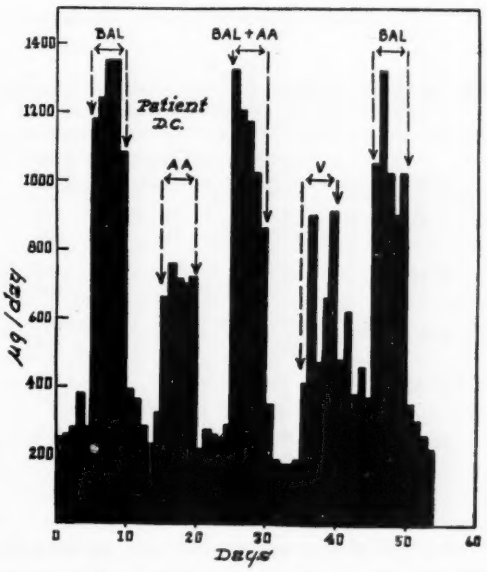


FIG. 3. THE INFLUENCE OF B.A.L. (DIMERCAPTOPROPANOL), CASEIN HYDROLYSATE (AA), AND "CALCIUM VERSENATE" (V, GIVEN INTRAVENOUSLY) ON THE EXCRETION OF COPPER IN THE URINE

For experimental details, consult the text.

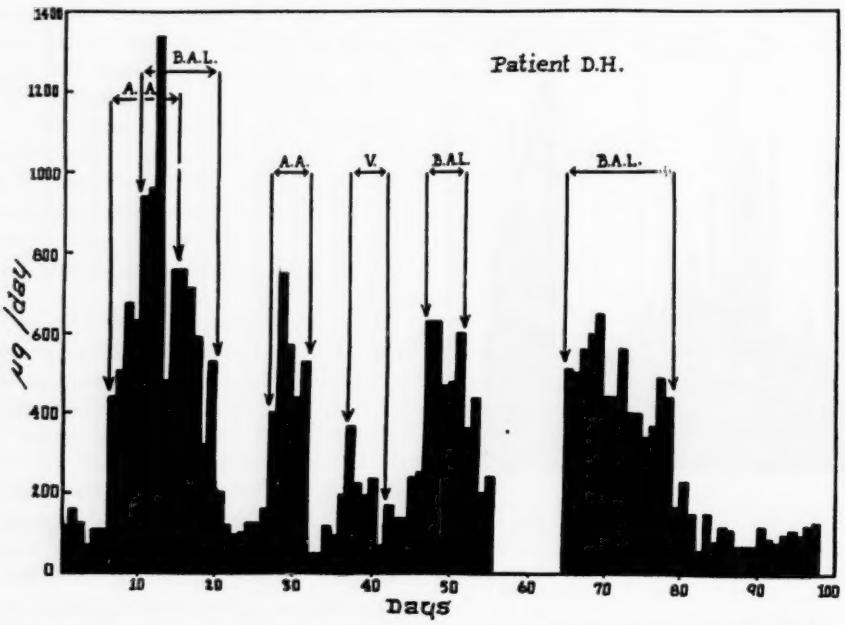


FIG. 4. THE INFLUENCE OF CASEIN HYDROLYSATE (AA), B.A.L. (DIMERCAPTOPROPANOL), AND "CALCIUM VERSENATE" (V, GIVEN INTRAVENOUSLY) ON THE EXCRETION OF COPPER IN THE URINE

For experimental details, consult the text.

TABLE IX

*The influence of the prolonged administration of potassium sulfide on the plasma and urine copper levels*

| Patient | Duration of therapy *months* | Plasma copper *μg/100 ml.* | Urine copper *μg/day* |
|---|---|---|---|
| D. C. | 0 | 71 | 415 |
| | 12 | 71 | 408 |
| R. K. | 0 | 43 | 185 |
| | 7 | 39 | 100 |
| J. S. | 0 | 57 | 181 |
| | 11 | 57 | 260 |
| W. K. | 0 | 43 | 297 |
| | 8 | 59 | 440 |
| M. L. | 0 | 36 | 837 |
| | 4 | 41 | 762 |

## DISCUSSION

The total plasma copper level was significantly reduced in all of the patients in this study. This has also been observed by Bearn and Kunkel (12, 13, 38) whose reports appeared while these investigations were in progress. In addition, it has been demonstrated in this study that there is an absolute increase in the amount of direct-reacting copper, with the result that there is an even more striking reduction in the indirect-reacting fraction (ceruloplasmin) than is indicated by the reduced total plasma copper level. This adds support to the more direct observations of Scheinberg and Gitlin (16) that there is a marked deficiency of ceruloplasmin in patients with Wilson's disease.[4] The increase in the direct-reacting fraction is probably a manifestation of the extremely high tissue levels of copper and probably reflects the increased turnover of copper from the gastro-intestinal tract to the tissues and from the tissues to the excretory organs, the kidney, liver, and intestine. Previous studies have shown that the direct-reacting fraction is concerned in the transportation of copper (15). The copper in the direct-reacting fraction is not dialyzable *in vitro* and the evidence available suggests that this

[4] Recent studies in our laboratory (H. Markowitz) indicate that there is a high degree of correlation between the indirect-reacting fraction of plasma copper and ceruloplasmin determined immunologically.

copper may be bound to serum albumin (17). The observation that the concentration of copper in the spinal fluid is about one-half the value of the direct-reacting fraction in plasma suggests that the direct-reacting copper is not completely dialyzable *in vivo*. However, that a portion of it is dialyzable is suggested by the finding that both the direct-reacting fraction in the plasma of the patients and the concentration of copper in their spinal fluids was three times greater than the mean values found in the control subjects.

It is of considerable interest that there was a marked reduction in the indirect-reacting fraction of plasma copper in the patient (Dar. H.) with relatively early Wilson's disease, in the absence of an increase in the excretion of amino acids in the urine. Stein, Bearn, and Moore (20) studied one patient with well-advanced Wilson's disease with hypocupremia in whom there was no significant aminoaciduria. These observations suggest that the deficiency of ceruloplasmin may precede development of aminoaciduria, from which it may be suspected that the disturbance in copper metabolism may be the primary defect in this disease rather than the disturbance in amino acid metabolism. Earlier observations (3, 9, 10) of a normal or elevated plasma copper level with which our observations and those of Bearn and Kunkel are not in agreement might be explained by assuming that the copper analyses were in error, or, more likely, the direct-reacting fraction was increased to such a degree as to completely obscure the decrease in ceruloplasmin.

The observation that the tissue copper level is increased and that patients with Wilson's disease receiving a normal diet are in positive copper balance, must mean that in hepatolenticular degeneration, either the absorption of copper from the gastro-intestinal tract is increased, or the excretion of copper from the body is decreased. Theoretically, a decreased excretion of copper could result from an increased avidity of the tissues for copper or from a failure of one or more of the main excretory routes for copper to function properly. From the available evidence it cannot be stated dogmatically which of these mechanisms might be at fault in Wilson's disease. In normal subjects the main excretory route for copper is through the bile (39). In addition, copper may be excreted directly through the intestinal wall. The observation in one of our patients that the concentration of copper in the bile removed directly from the gall-bladder at autopsy was normal, together with the finding of Denny-Brown and Porter (6) of a normal concentration of copper in the bile in three of their patients, suggests that the biliary copper excretion is normal. Measurement of the amount of copper excreted directly through the intestinal wall has not been possible. That the affinity of the tissues for copper is increased, seems rather unlikely since the excretion of copper in the urine is increased and since the tissue copper can be readily mobilized by B.A.L. or casein hydrolysate. The administration of these agents to normal subjects is not associated with an increase in the excretion of copper in the urine (10, 13).

From the above observations and deductive reasoning it would seem logical to conclude that in patients with Wilson's disease, the absorption of copper from the gastro-intestinal tract is increased. This conclusion is in agreement with that of Zimdahl, Hyman, and Cook (40). However, it must be admitted that the studies to date have not completely eliminated the possibility that the excretion of copper into the intestinal tract is decreased. In fact, Bearn and Kunkel (17) have observed that the fecal excretion of intravenously administered copper$^{64}$ is diminished in patients with Wilson's disease as compared with normal subjects.

Our determinations of the concentration of copper in the tissues of the one patient who died confirm the earlier observations (1, 3, 4) that in Wilson's disease there is an accumulation of copper in the tissues. The concentration of copper was increased about 10-fold in all of the tissues, except heart muscle, skeletal muscle, lung, and erythrocytes. This was true in spite of the fact that the patient had been given several courses of B.A.L., casein hydrolysate, and "Calcium Versenate" (Figure 4) four months prior to his death. It should be noted that these results are at variance with those of Palmer, Drew, and Chenoweth (2) who found essentially normal tissue copper levels in a patient with hepatolenticular degeneration who had been given four courses of B.A.L. therapy.

It is tempting to conclude, as have others (13), that the pathological lesions in the liver and brain,

and the functional renal defect, are the consequence of the accumulation of excessive amounts of copper in these organs. That copper may have such an effect is suggested by the cirrhosis of the liver which has been produced experimentally in rabbits by the administration of large amounts of copper (41) and the cirrhosis which has been described in sheep under natural conditions which are conducive to the retention of copper in the tissues (42, 43). Furthermore, in this laboratory it was possible to damage the renal tubular epithelium of rats by the administration of excessive amounts of copper (44). In further support of this concept is the observation that, after the mobilization of copper from the tissues, clinical improvement occurred in some patients (6). In one of our patients (D. C.) the degree of improvement has been extremely impressive. Prior to the first course of B.A.L. and potassium sulfide therapy, the patient was frankly psychotic and tremors and rigidity were marked. Approximately a month after therapy was initiated, the patient showed no mental abnormalities at all, the rigidity had entirely disappeared and the tremors were much less severe. With each subsequent course of therapy, the neurologic state has improved. That all patients do not improve, or that the improvement is only partial and temporary is not at all surprising, if consideration is given to the quantitative aspects of the problem. According to our findings, patients with this disease may be in positive copper balance to the extent of 0.5 to 2.5 mg. of copper per day. The chelating agents, in the amounts which have been used, mobilize about 0.5 to 1.0 mg. per day. In one patient the influence of the agents was not great enough to establish a negative balance. Furthermore, these agents are not given continuously and positive balance is reestablished as soon as therapy is discontinued. When these facts are taken into consideration, it is more surprising that a few of the patients improve temporarily, rather than that all of the patients are not benefitted. To produce marked improvement it may be expected that therapy with the available agents will have to be more energetic and more continuous than heretofore or that more effective agents will have to be found.

With our present knowledge and the agents available, the most effective combination of drugs and schedule of therapy might be outlined as follows. The patients should be given as high a protein diet as possible. In this way, the aminoaciduria will be increased, and greater quantities of copper will be excreted in the urine. In addition, the patients should be given 20 mg. of potassium sulfide with each meal in order to decrease the absorption of copper. Five day courses of B.A.L. (2.5 mg. per kg. twice daily) or casein hydrolysate (one liter of a 5 per cent solution per day) should be given every 10 days until maximum improvement occurs. Ideally, the above regimen should be maintained continuously.

From the data presented in this paper a working hypothesis to explain the pathogenesis of Wilson's disease may be outlined. It is anticipated that as further data accumulate, this hypothesis will require modification. Furthermore, it must be conceded that this hypothesis does not explain adequately all of the observations which have been made to date.

According to this hypothesis, the primary defect in hepatolenticular degeneration is a congenital inability to synthesize ceruloplasmin. As a result of the deficiency of ceruloplasmin, the absorption of copper from the gastro-intestinal tract is increased and the direct-reacting fraction of plasma copper is increased. The deposition of excessive amounts of copper in the brain and liver causes the characteristic pathologic lesions in these organs. The accumulation of copper in the kidneys produces a functional impairment in the reabsorption of certain amino acids and peptides. Aminoaciduria and peptiduria result. As a consequence of the accumulation of copper in the tissues, copper is chelated by amino acids and peptides and is excreted in increased amounts in the urine.

The above concept is in harmony with that of Scheinberg and Gitlin (16) and with that of Bearn and Kunkel (12, 13, 17).

This hypothesis assigns to ceruloplasmin an important role in regulating the amount of copper absorbed. It does not explain how this function is achieved. Admittedly, it is difficult to visualize how ceruloplasmin regulates absorption since the copper in ceruloplasmin is apparently not in equilibrium with the direct-reacting copper. Perhaps as a result of the deficiency of ceruloplasmin, cop-

per is absorbed and retained in excessive quantities in an effort to make more ceruloplasmin, or a precursor of ceruloplasmin might accumulate.

From these speculations it is apparent that information is very much needed concerning the form in which copper is deposited in the tissues in Wilson's disease. The possibility must not be overlooked that ceruloplasmin may not be the only copper protein which is deficient. Studies concerning other copper proteins such as hepatocuprein, tyrosinase, and butyryl Co A dehydrogenase should be undertaken. Our observation that the erythrocyte copper level is normal, and the fact that anemia is not consistently observed in patients with this disorder suggests, although it does not prove, that the copper protein of the red cells is intact, and that, therefore, the impairment of copper protein synthesis does not involve all of the copper proteins.

It is of interest to speculate on one additional observation. A severe hemolytic anemia of sudden onset preceded by one year the diagnosis of Wilson's disease in one patient (Dar. H.). In three of Wilson's patients (45) an attack of jaundice antedated the onset of the clinical syndrome. Others have reported a similar event. A disease, known as "Enzootic Jaundice," has been described in sheep in Australia (42, 43) and has been produced experimentally (46). In this condition, for reasons which are not understood, the tissues retain excessive quantities of copper. The sheep suffer no apparent ill health until suddenly there is a "catastrophic liberation" of copper into the blood stream. The plasma level of copper increases to values of more than ten times normal. Severe hemolytic anemia, jaundice, and hemoglobinuria follow. Death frequently supervenes as a result of severe renal failure. If the animals survive and suffer two or three such crises, cirrhosis of the liver then results. By comparison it is possible that such crises may occur in patients with Wilson's disease. The severe hemolytic anemia in our patient may have been related to such an event. This suggestion is made in the hope that it will encourage investigators who should have the opportunity to study in detail courses of jaundice occurring in patients with this disease.

SUMMARY

A. Various aspects of copper metabolism have been studied in seven patients with hepatolenticular degeneration. The following abnormalities have been demonstrated:

1) A decrease in the plasma total copper level;

2) An increase in the direct-reacting fraction of plasma copper;

3) a decrease in the indirect-reacting fraction (ceruloplasmin) of plasma copper;

4) an increase in the concentration of copper in the spinal fluid, all of which was direct-reacting;

5) a large increase in the concentration of copper in all of the tissues studied, except heart muscle, skeletal muscle, lung, and erythrocytes;

6) a state of positive copper balance; and

7) an increased excretion of copper in the urine.

B. A patient is described with early Wilson's disease, in whom the indirect-reacting fraction of plasma copper was markedly depressed in the absence of an increased excretion of amino acids in the urine.

C. As a tentative hypothesis to explain the observations, it is proposed that in such patients there is a congenital deficiency in the formation of ceruloplasmin. As a consequence, the absorption of copper is increased and copper accumulates in the tissues. As a result of the accumulation of copper in the brain, liver, and cornea, the characteristic pathological lesions of the disease appear. The accumulation of copper in the kidney causes a functional impairment in the reabsorption of certain amino acids and peptides, and aminoaciduria and peptiduria result. With the accumulation of copper in the tissues, copper is chelated by amino acids and peptides and is excreted in increased amounts in the urine. The increase in the direct-reacting fraction of copper in the plasma is a manifestation of the extremely high tissue levels of copper and reflects the increased turnover of copper between the gastro-intestinal tract, the tissues, and the excretory routes.

ACKNOWLEDGMENTS

We are indebted to Miss Helen Ashenbrucker, Miss Doris Kurth, and Mrs. Jean Van Dilla for technical assistance. Miss Ruth Gunning gave valuable help in the arrangement of the diets. The nursing services of Mrs.

Gladys Rittenmeyer, Mrs. Ula Clark, and Mrs. Helen Huebner are gratefully acknowledged.

The electrophoretic analyses of the serum proteins were performed for us by Dr. Emil Smith and Dr. J. I. Routh. The "Calcium Versenate" was kindly supplied by Dr. Norman W. Karr, Riker Laboratories, Inc., Los Angeles, California; the "Amigen" by Warren M. Cox, Jr., Ph.D., Mead Johnson and Company, Evansville, Indiana; and the B.A.L. by John H. Brewer, Ph.D., Hynson, Westcott and Dunning Pharmaceutical Laboratory, Baltimore, Maryland.

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# CHANGES IN PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS DURING THE INTRAVENOUS ADMINISTRATION OF ACTH. I. A TEST OF ADRENOCORTICAL CAPACITY IN THE HUMAN [1]

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(Submitted for publication April 7, 1954; accepted July 15, 1954)

The recognition of the important role played by the adrenal cortex in normal physiology and its significance in withstanding "stressful situations" has led to the development of many tests to measure its functional state and capacity in the human. Most of these tests rely on measurements of phenomena which are dependent on the effect of adrenocortical secretions on some special metabolic process, and do not involve the direct measurement of adrenocortical steroids. The frequent discrepancies between the clinical picture and these indirect tests indicate that they are not always reliable indices of adrenal function.

Recently, Thorn, Goetz, Streeten, Dingman, and Arons (1) have devised a test for the quantitative estimation of adrenal function based on the determination of butanol-soluble 17,21-dihydroxy-20-ketosteroids in the urine. Adrenal capacity is estimated by the increase in urinary excretion of these compounds after intravenous administration of adrenocorticotrophic hormone (ACTH). While this technique is a considerable improvement over the indirect methods, measurements based on the urine have several disadvantages. If adrenal function or capacity must be determined over short periods of time, the technical difficulties of obtaining quantitative urine samples at frequent intervals are great. Alterations in renal function and steroid metabolism may also interfere with interpretation in terms of the internal hormonal environment. The development of a method for measuring 17-hydroxycorticosteroids in plasma (2) has made it possible to study adrenocortical function and its response to ACTH more directly.

In a previous paper from our laboratory the response of the adrenal cortex was measured after the rapid intravenous injection of a single dose of ACTH (3). It was stated in that paper that "the immediate injection of a single dose of ACTH was selected for its simplicity, with the reservation that such a technique, although valuable as a measure of the immediate ability of the gland to respond to stimulation, would be less significant in the assessment of the capacity of the gland to resynthesize and elaborate additional hormones, once available stores had been exhausted. A more prolonged stimulation, such as intravenous infusion of ACTH over a period of many hours, would test this aspect of glandular function more effectively." The present paper describes the effects of prolonged intravenous infusion of ACTH on plasma 17-hydroxycorticosteroid levels in a group of normal resting individuals. From these studies a standard method of testing adrenal cortical capacity has been developed.

## METHODS

The studies were performed on 39 normal subjects (33 males, 6 females) and six selected patients with adrenal insufficiency. All tests were begun between 8 and 9 A.M. and all the subjects were in the fasting state. The ACTH was given by continuous intravenous infusion in 500 ml. of normal saline or 5 per cent glucose in water.

[1] This investigation was supported in part by research grants A-29(C2) and A-2(C7) from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service; by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under contract No. DA-49-007-MD-136; and by a grant from Armour and Company.

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No subject was studied more frequently than every ten days. Most of the ACTH tests were performed with the same lot number (K56409) of "crude" ACTH (Armour), 25 I.U. being given over 6 hours. Some of the tests were performed with other ACTH preparations. The H.P. ("High Potency") Armour preparation is obtained by passage through oxycellulose and countercurrent distribution. Wilson ACTH (lot number 58792) is obtained by oxycellulose passage, as is the preparation obtained from Dr. Astwood. The ACTH obtained from Dr. Li (lot number L2397BP) had an activity of 80 I.U. per mgm. Corticotropin "Nyco" (lot number 034060) was obtained from the pituitary glands of whales and prepared by Nyegaard and Co., Norway. A few tests were done with Upjohn's ACTH (lot number CB243ZF).

Plasma 17-hydroxycorticosteroids were determined by modification (4) of the method of Nelson and Samuels (2). It should be pointed out that this method determines cortical steroids with a 17,21-dihydroxy-20-ketone group and includes such compounds as hydrocortisone, cortisone, and Reichstein's substance S. The method determines the hydrogenated forms of these steroids but not their water soluble conjugates. Although many substances in plasma will give the Porter-Silber reaction the material present after chloroform extraction and chromatography on florosil appears to be mostly hydrocortisone, particularly after ACTH. A detailed discussion of this problem and possible interfering non-steroidal substances is beyond the scope of this report but the frequent occurrence of undetectable levels in patients with adrenal insufficiency, two examples of which are presented here, is presumptive evidence of its specificity. Certain data concerned with the specificity of the method have been published previously (5, 6). A detailed study of the recoveries obtained, the character of steroids in human and dog plasma giving the reaction, and the possibility of interfering non-steroidal substances in plasma is being prepared for publication. Eosinophil counts were done by the method of Randolph (7).

## RESULTS

In order to test adrenocortical capacity, the amount of ACTH administered and the period of time during which such ACTH is given are of paramount importance. The effects of the intravenous infusion of different amounts of ACTH on the 17-hydroxycorticosteroid levels in the plasma of normal individuals are compared in Table I. The time of infusion was 6 hours in this series. As little as 1 I.U. and possibly 0.1 I.U. of ACTH had an effect in raising the levels of 17-hydroxycorticosteroids in the blood plasma. These increases were, however, submaximal. What appeared to be maximal stimulation of the adrenal cortex was induced by 15 to 25 I.U. of ACTH. There was no significant difference between the increase in plasma 17-hydroxycorticosteroids during the administration of 50 I.U. of ACTH and that during 25 I.U. (Tables I, II, and Figure 1). A dose of 25 I.U. of ACTH was, therefore, selected as giving maximal stimulation of the adrenal cortex as measured by the increase in plasma 17-hydroxycorticosteroid levels in 6 hours.

TABLE I

*Comparison of the changes in the levels of plasma 17-hydroxycorticosteroids when different doses of ACTH were infused intravenously over a 6-hour period* *

| Subject | Amount of ACTH I.U. | Plasma 17-OH-CS levels γ/100 ml. 0 hr. | 2 hrs. | 4 hrs. | 6 hrs. |
|---|---|---|---|---|---|
| A | 1/10 | 4 | 17 | 17 | 21 |
| A | 25 | 5 | 23 | 27 | 30 |
| A | 25 | 8 | 30 | 43 | 50 |
| B | 1 | 8 | 15 | 20 | 22 |
| B | 10 | 11 | 24 | 32 | 29 |
| B | 25 | 6 | 27 | 32 | 40 |
| B | 25 | 7 | 20 | 26 | 30 |
| C | 15 | 10 | 30 | 40 | 46 |
| C | 25 | 12 | 26 | 32 | 40 |
| C | 25 | 4 | 21 | 22 | 23 |
| D | 50 | 5 | 20 | 32 | 38 |
| D | 25 | 8 | 30 | 36 | 48 |
| E | 50 | 17 | 36 | 39 | 40 |
| E | 25 | 11 | 34 | 36 | 45 |

* Tests were run at two to three different dose levels in the same individual.

A series of experiments was performed to determine the rate of infusion of ACTH which would give a maximal rate of increase in plasma 17-hydroxycorticosteroids. A total of 25 I.U. of ACTH was infused over periods of 2, 4, 6, and 8 hours. As seen in Table III, there was no significant difference in the rates of increase of 17-

TABLE II

*Levels of 17-hydroxycorticosteroids during the intravenous infusion of 50 I.U. ACTH over 6 hours*

| Subject | Plasma 17-OH-CS levels γ/100 ml. 0 hr. | 2 hrs. | 3 hrs. | 4 hrs. | 6 hrs. |
|---|---|---|---|---|---|
| D | 5 | 20 | | 32 | 38 |
| M | 11 | 40 | | 50 | 63 |
| E | 17 | 36 | | 39 | 40 |
| O | 14 | | 30 | | 40 |
| P | 10 | | 33 | | 42 |
| Q | 15 | | 35 | | 48 |
| R | 16 | | | | 51 |
| S | 15 | | | | 48 |
| T | 10 | | | | 50 |
| Mean | 13 | | | | 47 |
| Mean, 25 I.U. (Figure 1) | 10 | | | | 42 |

TABLE III

*The effects of 25 I.U. ACTH over different periods of time upon plasma levels of 17-hydroxycorticosteroids*

| Subject | Length of infusion (*hrs.*) | Plasma 17-OH-CS levels γ/100 ml. 0 hr. | 2 hrs. | 4 hrs. | 6 hrs. | 8 hrs. |
|---|---|---|---|---|---|---|
| M | 2 | 20 | 29 | | | |
| N | 2 | 10 | 33 | | | |
| O | 4 | 5 | 26 | 37 | | |
| P | 4 | 3 | 20 | 28 | | |
| Q | 8 | 10 | 24 | 43 | 44 | 46 |
| A | 8 | 14 | 34 | 40 | 62 | 55 |
| A | 6 | 8 | 30 | 43 | 50 | |
| A | 6 | 5 | 23 | 27 | 30 | |
| B | 8 | 10 | 25 | 34 | 34 | 23 |
| B | 6 | 6 | 27 | 32 | 40 | |
| B | 6 | 7 | 20 | 26 | 30 | |
| C | 8 | 10 | 28 | 25 | 31 | 31 |
| C | 6 | 12 | 26 | 32 | 40 | |
| C | 6 | 4 | 21 | 22 | 23 | |

hydroxycorticosteroids in the plasma among the different rates of infusion. Furthermore, when the dose was spread over 8 hours, the level at the end of the infusion did not differ significantly from that at 6 hours.

The means and standard deviations of the response of the plasma 17-hydroxycorticosteroid levels to the intravenous infusion of 25 I.U. of ACTH over 6 hours in 39 normal resting subjects are shown in Figure 1. It will be observed that the standard deviation of values increases proportionately with the mean.

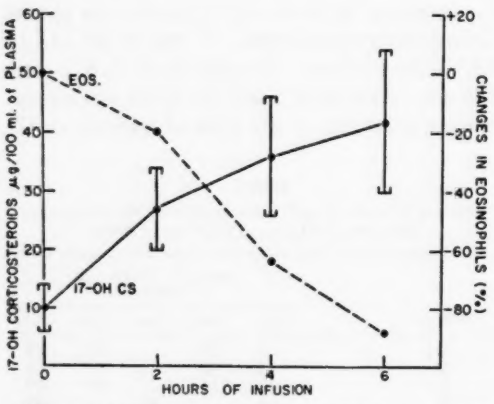


FIG. 1. PLASMA 17-HYDROXYCORTICOSTEROID LEVELS DURING THE INTRAVENOUS INFUSION OF 25 I.U. OF ACTH

The mean and standard deviation of the response of these levels in 39 normal subjects are shown. The changes in the eosinophilic leukocytes are correlated with the changes in the steroid levels.

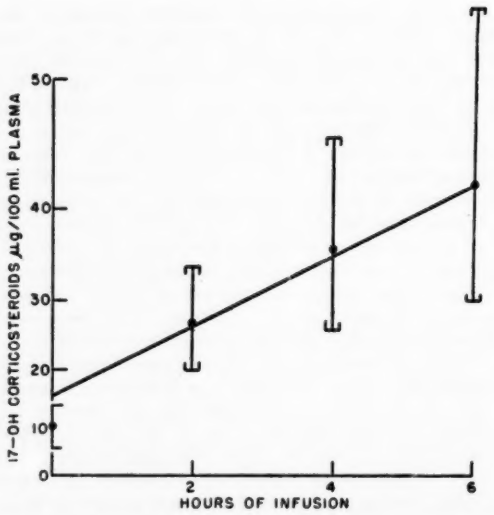


FIG. 2. THE MEAN AND STANDARD DEVIATION OF PLASMA 17-HYDROXYCORTICOSTEROID LEVELS DURING THE INFUSION OF 25 I.U. OF ACTH PLOTTED ON AN INVERSE LOGARITHMIC SCALE

In Figure 2 the rate of increase of plasma 17-hydroxycorticosteroids during the intravenous infusion of 25 I.U. of ACTH over 6 hours is plotted on an inverse logarithmic scale. Except for the initial 2-hour period, the mean values all fall along a straight line. This would be the result expected if there were constant input and removal proportionate to the level attained. In other work it has been demonstrated that once initial equilibrium has been achieved, 17-hydroxycorticosterone and cortisone are removed from the blood stream at a rate proportional to concentration (8, 9). During the first period, however, there is evidence of a more rapid increase. This may be due to small amounts of steroids which can be quickly mobilized for secretion. The 6-hour infusion permits the determination of three values at 2-hour intervals which are beyond this initial point, at which time a maximal constant rate of secretion seems to have been established. The shorter infusions were discarded because of possible confusion between initial output and that which could be maintained.

In Figure 3 is shown a representative curve of the plasma 17-hydroxycorticosteroids during and following the continuous infusion of ACTH over many hours. It is apparent that the levels reached

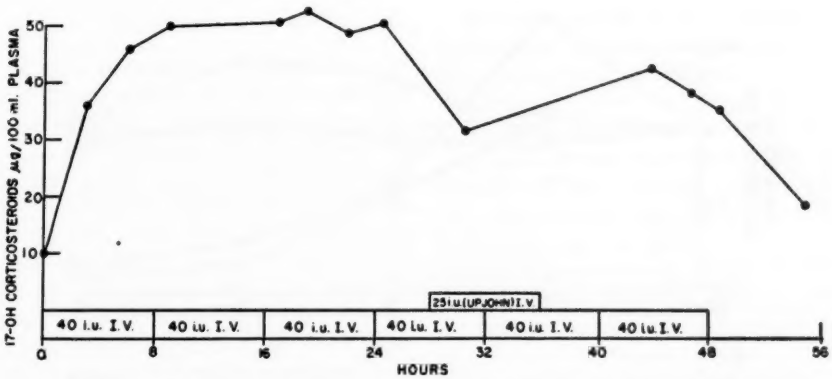


FIG. 3. PLASMA 17-HYDROXYCORTICOSTEROID LEVELS DURING THE CONTINUOUS INFUSION OF ACTH( 40 I.U. WILSON ACTH q.8 H.) OVER 48 HOURS

Between the 28th and 36th hour an additional 25 I.U. of ACTH (Upjohn) was infused. The explanation of the apparent fall in level during this period is unkown at present.

a plateau after 6 hours which was maintained as long as the ACTH was given. The steroid levels returned to the normal range within 6 hours after cessation of the infusion. Halfway through the experiment the subject was given an additional 25 I.U. of ACTH (Upjohn) in addition to the continuous infusion that he was receiving. This was done in order to ascertain whether maximal adrenal stimulation was being obtained by the amount of ACTH which was given; if not, the additional ACTH should have raised the plasma 17-hydroxycorticosteroids further. Instead, the plasma sample at the end of this period gave a lower value.

It would appear, therefore, that the infusion of 25 to 30 I.U. in 6 hours achieves a stimulation of the adrenal cortex which is maximal. It is also a test of the capacity of the adrenal cortex to maintain production of cortical steroids having a 17,21-hydroxy-20-ketone group.

The response of the same subject to a standard ACTH test on different occasions showed considerable variation and is illustrated in Table IV. This variation in response does not seem to depend on the control level of plasma 17-hydroxycorticosteroids.

To determine whether the type of preparation would affect the test, six different preparations of ACTH were infused in the standard manner. It appeared that, in the dose given (25 I.U.), neither the animal source nor the difference in the technical procedures of preparing ACTH had any effect on its capacity to elicit maximal adrenocortical stimulation. Apparently, the U.S.P. assay is satisfactory for evaluation of the potency of ACTH administered intravenously in the human.

The validity of the procedure as a measure of adrenocortical capacity was tested in subjects with clinical symptoms of adrenal insufficiency (Figure 4). In each case there was little or no rise in the plasma 17-hydroxycorticosteroid levels fol-

TABLE IV

*Plasma levels of 17-hydroxycorticosteroids following the intravenous infusion of 25 I.U. of ACTH over 6 hours in the same subject on different occasions*

| Subject | Plasma 17-OH-CS levels γ/100 ml. | | | |
|---|---|---|---|---|
| | 0 hr. | 2 hrs. | 4 hrs. | 6 hrs. |
| A | 5 | 23 | 27 | 30 |
| A | 8 | 30 | 43 | 50 |
| B | 6 | 27 | 32 | 40 |
| B | 7 | 20 | 26 | 30 |
| C | 12 | 26 | 32 | 40 |
| C | 4 | 21 | 22 | 23 |
| F | 8 | 26 | 28 | 49 |
| F | 10 | 30 | 54 | 60 |
| G | 9 | 28 | 40 | 46 |
| G | 3 | 21 | 22 | 23 |
| G | 12 | 26 | 32 | 40 |
| H | 4 | 23 | 29 | 32 |
| H | 17 | 31 | 41 | 56 |
| I | 10 | 20 | 25 | 38 |
| I | 12 | 25 | 26 | 39 |
| I | 12 | 26 | 43 | 40 |
| J | 4 | 22 | 25 | 20 |
| J | 6 | 32 | 40 | 43 |
| K | 6 | 27 | 33 | 40 |
| K | 8 | 20 | 26 | 30 |
| L | 8 | 18 | 28 | 28 |
| L | 8 | 26 | 50 | 43 |

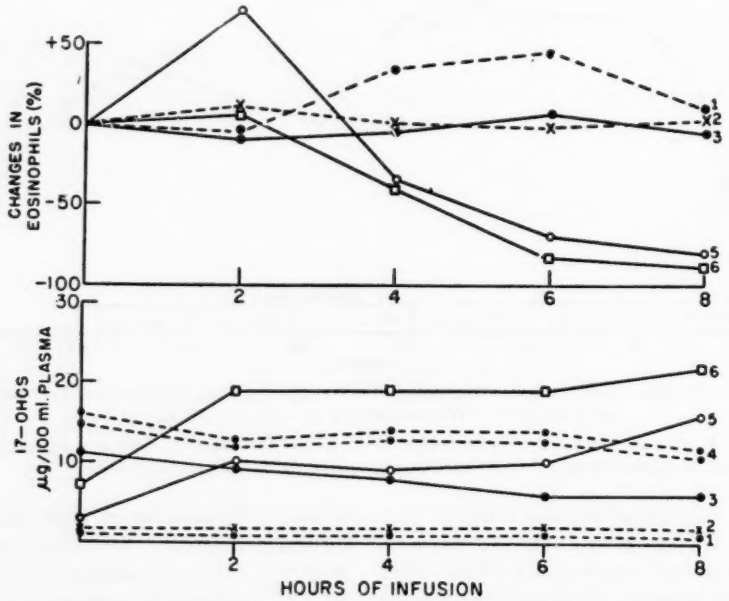


FIG. 4. CHANGES IN PLASMA 17-HYDROXYCORTICOSTEROID LEVELS AND CIRCULATING EOSINOPHILS IN SIX PATIENTS WITH ADRENAL INSUFFICIENCY DURING THE I.V. INFUSION OF 25 I.U. OF ACTH OVER 8 HOURS

lowing the intravenous infusion of 25 I.U. of ACTH over 6 to 8 hours. It is presumed that in the patients with demonstrable 17-hydroxycorticosteroid levels in plasma, the small amount of adrenal cortex still functioning was stimulated almost maximally by endogenous ACTH. No demonstrable steroid levels were seen after the standard ACTH test in Addisonian patients with no measurable plasma 17-hydroxycorticosteroid levels beforehand (Cases 1 and 2, Figure 4).

In each subject showing a significant rise in 17-hydroxycorticosteroid levels following ACTH administration, there was a definite effect on the levels of circulating eosinophils (Cases 5 and 6, Figure 1). Although the largest rise in 17-hydroxycorticosteroids occurred during the first 2 hours of ACTH infusion, a significant decrease in eosinophils of over 50 per cent did not occur until 4 hours after the beginning of the infusion (Figures 1, 4, and Table V). At the end of the infusion of ACTH the eosinophil count was lowest, while the plasma 17-hydroxycorticosteroids were usually highest. It is interesting to observe that in subjects with adrenocortical insufficiency a significant drop in the eosinophils was found only when the subject showed an increase of plasma 17-hydroxycorticosteroids following the standard test for adrenocortical capacity (Figure 4).

The usefulness of the response of the circulating eosinophils is, however, limited under "stressful" conditions. As can be seen from Table V, there were definite increases in the plasma levels of 17-hydroxycorticosteroids following the standard ACTH test in a case where the eosinophils were too low to give any indication of changes in corticosteroid levels.

TABLE V

*Plasma 17-hydroxycorticosteroid levels and blood eosinophils following the administration of 25 I.U. of ACTH intravenously over 6 hours in a patient before and after a major surgical operation (removal of the gallbladder, general anesthesia)*

| Time after beginning of ACTH adm. | One week before surgery | | Immediately after surgery | |
|---|---|---|---|---|
| | 17-OH-CS in plasma γ/100 ml. | Eosinophils per mm.³ | 17-OH-CS in plasma γ/100 ml. | Eosinophils per mm.³ |
| 0 | 9 | 360 | 60 | 3 |
| 2 | 37 | 334 | 78 | 0 |
| 4 | 50 | 106 | 107 | 0 |
| 6 | 52 | 12 | 96 | 0 |

## DISCUSSION

From the data presented it is evident that the intravenous administration of 25 I.U. ACTH over 6 hours serves as a test of adrenocortical capacity. The demonstration of diminished capacity by this test in patients who had shown symptoms of Addison's disease points to the reliability of the test. It is also apparent from the data that the increment in plasma 17-hydroxycorticosteroids during the first two hours may exceed the rate expected on the basis of constant input and logarithmic removal, and, therefore, probably includes release of readily mobilized precursor as well as maximal rate of synthesis. Continuation of the stimulation with ACTH beyond the 6-hour period results in little further elevation of 17-hydroxycorticosteroid levels and is, therefore, unnecessary for the evaluation of the adrenocortical capacity.

The test described offers some advantages over those presently in existence. The number of eosinophils cannot always be used as a reliable index of adrenocortical function since it is influenced by other factors (10). The urinary 17-ketosteroids arise from other sources besides the adrenal cortex. In addition, all measurements on urine involve the function of the kidneys. Abnormalities in these organs will influence the rate of excretion of steroids in relation to blood levels, and may, therefore, lead to erroneous results after ACTH stimulation.

In the procedure outlined here only the change in levels of free 17-hydroxycorticosteroids during ACTH administration is measured. The steroids excreted in the urine are largely conjugated metabolic products which are not determined in this method. Under these conditions if changes in the levels of conjugated compounds due to renal impairment occurred they would have little effect on the test. The present method, therefore, overcomes a number of errors involved in past procedures for measuring adrenal capacity. The amounts of blood required for the determination of plasma 17-hydroxycorticosteroids can be repeatedly withdrawn at short intervals. In addition, the administration of ACTH and its demands on the adrenal cortex can be evaluated concomitantly with the ACTH administration.

In conditions where the adrenocortical steroid environment is undergoing frequent changes, it is difficult to evaluate the influence of infused ACTH. Fluctuations in the amount of 17-hydroxycorticosteroids in the blood plasma may be due to endogenous changes rather than to the effect of exogenous ACTH. It is felt, therefore, that the test for adrenocortical capacity as described in this paper may best be applied in the normal resting human, in patients with adrenal insufficiency, and in pathological conditions where a stable adrenocortical environment has been established. Results under other circumstances must be interpreted with caution, and are of more qualitative than quantitative significance.

## SUMMARY

1. Utilizing changes of 17-hydroxycorticosteroid levels in blood plasma following the intravenous administration of 25 I.U. ACTH over 6 hours, a test has been devised for evaluating adrenocortical capacity in the normal resting human.

2. When 50 I.U. ACTH were infused over the same period there was no significant increase in the rate of change of the plasma 17-hydroxycorticosteroids.

3. Individuals who showed symptoms of Addison's disease showed a decreased response even when the levels of 17-hydroxycorticosteroids before ACTH infusion were within normal limits.

4. Advantages and limitations of the test as a measure of adrenocortical capacity are discussed.

## ACKNOWLEDGMENTS

We are grateful to Dr. E. Hays of Armour and Co., Dr. R. Klein of Wilson and Co., Dr. E. Astwood, Dr. C. Li, Dr. M. Kuizenga of Upjohn and Co., and Dr. O. Bjørnson of Nyegaard and Co., for generous supplies of various ACTH preparations.

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# THE EFFECTS OF SURGERY ON THE BLOOD LEVELS AND METABOLISM OF 17-HYDROXYCORTICOSTEROIDS IN MAN [1, 2]

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(Submitted for publication April 7, 1954; accepted July 15, 1954)

The role of the adrenal cortex in the maintenance of homeostasis has been reviewed extensively by Sayers (1). It is evident from the experimental data published, as well as from observations on patients with adrenal insufficiency, that the adrenal cortex is necessary for the maintenance of life when untoward conditions are present in the environment. Such conditions have been referred to as "stresses" by Selye (2) and many others. "Stress," however, is such a nonspecific term that its use may lead to confusion. It has become apparent from studies in our laboratory that various noxious agents may have different effects on the circulating levels of 17-hydroxycorticosteroids. Consequently, the concept that all types of "stress" have a similar effect on adrenal secretion and adrenal steroid metabolism does not seem to be justified.

However, in a variety of such "stressful" circumstances (exposure to cold, trauma, histamine, *etc.*) in animals it has been demonstrated that a stereotyped series of events ensues which has been presumed to result from stimulation of the adrenal cortex by adrenocorticotrophic hormone (ACTH) (1, 3). In the adrenal cortex itself the changes have included depletion of ascorbic acid and cholesterol and increase in the weight of the gland. All of these are characteristic effects of ACTH administration. Such effects are not found after hypophysectomy.

It has also been demonstrated that certain metabolic changes which are similar to the effects which follow the administration of adrenocortical steroids are observable in both man and animals after trauma. A number of studies have shown, however, that although the presence of adrenal hormone is essential for the intensification of the adrenal-controlled metabolic processes following trauma, similar responses are produced in adrenalectomized animals maintained on constant doses of adrenal cortical extract, cortisone or hydrocortisone, where there can be no question of increased adrenal hormone secretion (4–6). Epinephrine, which Cannon (7) demonstrated long ago to be regularly increased after "stress" and trauma, appears not to produce a rise in plasma 17-hydroxycorticosteroid levels in the human but requires the presence of small amounts of adrenal cortical hormone for its adrenal-like metabolic effects to become manifest (8, 9).

These findings suggested that it might be important to evaluate the effect of the trauma of surgical operations on plasma levels of adrenal steroids and on their secretion. It has been demonstrated that the metabolic effects of surgery simulate in certain respects the effects of the administration of adrenal steroids (10, 11).

Sayers and Sayers (12) have presented evidence to support the hypothesis that the apparent stimulation of the adrenal cortex by "stress" might be the result of a compensatory response of the hypophysis to the utilization of circulating steroid by the "stressed" tissue. In this case one would expect that the 17-hydroxycorticosteroid level would first be decreased, stimulating the output of ACTH. Furthermore, the adrenal cortical capacity (ability to secrete additional amounts of

[1] Some of these data were presented at the Annual Meeting of the Society for Clinical Investigation, Atlantic City, New Jersey, May 5, 1953.

[2] This investigation was supported in part by research grants from the National Institutes of Health, U. S. Public Health Service and from Armour and Co. and in part by a contract (DA-49-007 MD-136) between the University of Utah and the Department of the Army.

[3] Scholar in Cancer Research of the American Cancer Society. The work presented in this paper was performed during the tenure of a Fellowship of the Dazian Foundation for Medical Research (1952–53).

[4] Fulbright and Baxter Fellow.

adrenal steroids) might be expected to be impaired because of the increased spontaneous output. In order to examine this hypothesis, we have used a test of adrenal cortical capacity in man. This test is based on the effect of a maximally stimulating intravenous dose of ACTH on the circulating steroid levels. The details of the technique and the results in normal individuals have been presented in detail in an earlier paper (13). The effect of surgical trauma on the rate of disappearance of intravenously administered hydrocortisone was also determined.

## METHODS AND MATERIALS

In patients undergoing surgery, blood samples were obtained by venesection when the patient arrived in the operating-room, following the induction of anesthesia, and at regular intervals during and following the surgical procedures. On certain patients control studies were performed at least one week before or four weeks after the operation.

All patients received the usual preoperative medications. These usually consisted of some barbiturate the night before and morphine and atropine on the morning of the operation.

Plasma 17-hydroxycorticosteroid levels were determined by a modification of the method of Eik-Nes, Nelson, and Samuels (14). The term 17-hydroxycorticosteroids, as used in this paper, is understood to refer to adrenal cortical steroids with a 17,21-dihydroxy-20-ketone group, such as cortisone, hydrocortisone, Reichstein's substance S, and the hydrogenated derivatives of these compounds. The method, however, does not measure the conjugated forms of these steroids in plasma. Circulating eosinophils were counted by the method of Randolph (15).

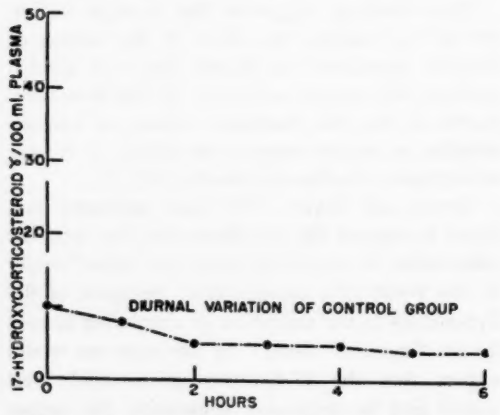


FIG. 1. DIURNAL VARIATION OF PLASMA 17-HYDROXYCORTICOSTEROIDS, BEGINNING AT 8 A.M. (0 HOURS), IN NORMAL SUBJECTS

Adrenal cortical capacity was tested by the intravenous infusion of maximally stimulating amounts of ACTH over a 6-hour period (13). Venous blood samples were drawn for determination of plasma 17-hydroxycorticosteroid levels before and at 2, 4, and 6 hours after the infusion was started.

The ability to remove excess hydrocorticosterone was determined by the infusion of 50 mg. of hydrocortisone (free alcohol) in saline solution containing a small amount of ethanol (16).

## RESULTS

### *Plasma 17-hydroxycorticosteroid levels in normal subjects and the diurnal variations of these levels*

During the progress of these experiments plasma 17-hydroxycorticosteroids were determined in 84 non-surgical subjects. The average 8 A.M. level was 13 $\mu$g per 100 ml. of plasma with a total range of 3 to 22 $\mu$g per 100 ml. of plasma. The average curve of diurnal variation in normal individuals from this series is shown in Figure 1. It will be observed that the level is high in the morning (8 A.M.) with a gradual decline during the day, as previously described (17).

### *Changes in plasma 17-hydroxycorticosteroids following anesthesia*

It will be noted in Table I that none of the 12 patients in whom 17-hydroxycorticosteoid levels were measured at 8 A.M. before the induction of anesthesia showed a plasma 17-hydroxycorticosteroid level above the normal upper limit of 22 $\mu$g per 100 ml. of plasma. The average is the same (13 $\mu$g per 100 ml.) as that observed in a larger series of normal controls (17). Thus, neither the apprehension of the approaching surgical procedure nor the underlying clinical conditions had a significant effect on the plasma levels of 17-hydroxycorticosteroids. Following the induction of anesthesia the levels of 17-hydroxycorticosteroids rose to an average of 19 $\mu$g per 100 ml. of plasma. Although the number of cases is small, it was interesting to note that the induction of general anesthesia (pentothal, cyclopropane, ether) was associated with an increase in the plasma 17-hydroxycorticosteroid levels in seven out of eight cases but that after induction of spinal anesthesia only one patient showed a higher level. Since this last value was higher than that found two

TABLE I

*Effect of anesthesia and surgery on plasma concentrations of 17-hydroxycorticosteroids*

| No. | Age and sex | Anesthesia | 17-OH corticosteroids (γ/100 ml.) after beginning of operation: Before anesthesia | After anesthesia | 1 hr. | 2 hrs. | 3 hrs. | 4 hrs. | 5 hrs. | 6 hrs. | Operation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 59 F | Pentothal and cyclo then ether | 15 | 23 | 26 | 47 | 52 | | 65 | | Cholecystectomy |
| 2 | 70 F | Pentothal and ether | 18 | 25 | 26 | | 40 | | | | Smith-Peterson nailing |
| 3 | 56 F | Pentothal and cyclo | 9 | 11 | 12 | 25 | 24 | 25 | | | Resection of lung |
| 4 | 72 F | Pentothal and cyclo | 18 | 25 | 31 | 40 | 38 | | | | Cholecystectomy |
| 5 | 84 M | Cyclo | 4 | 9 | 2 | 4 | 5 | | | | Cholecystectomy |
| 6 | 65 F | Pentothal and cyclo | 14 | 13 | | 40 | 40 | 34 | | 42 | Abdominal-perineal resection of carcinoma of colon |
| 7 | 80 M | Pentothal and cyclo | 12 | 33 | | 38 | | | | | Smith-Peterson nailing |
| 8 | 56 M | Pentothal cyclo and ether | 10 | 27 | | 43 | 40 | 35 | | | Gastrectomy (carcinoma of stomach) |
| 9 | 70 M | Spinal | 9 | 9 | | 26 | | | | | Prostatectomy |
| 10 | 16 F | Spinal | 6 | 6 | 20 | 20 | | | | | Appendectomy |
| 11 | 50 F | Spinal | 20 | 17 | 20 | 30 | | | | | Hemorrhoidectomy |
| 12 | 69 M | Spinal | 19 | 35 | | 27 | | | | | Suprapubic prostatectomy |
| | | Average | 13 | 19 | 20 | 31 | 34 | 33 | 65 | 42 | |
| | | General | | 21 | | | | | | | |
| | | Spinal | | 17 | | | | | | | |

hours later, during surgery, it may represent a technical error.

*Changes in plasma 17-hydroxycorticosteroid levels during surgery*

Table I shows the changes in the plasma 17-hydroxycorticosteroids which took place during surgery in each of 12 patients. A gradual rise in the levels occurred which was especially striking when compared to the usual decrease in levels of plasma 17-hydroxycorticosteroids which occurs during this period of the day.

Case No. 5 was an exception to the general pattern observed. This patient was an 84-year-old white male who came to the hospital with severe jaundice due to calculi in the common bile duct. A cholecystectomy and exploration of the common bile duct were performed under cyclopropane anesthesia. The operation lasted three hours. The patient had no complications during or after surgery. On the assumption that the lack of any rise in 17-hydroxycorticosteroids in this patient might have been due to adrenal insufficiency, the patient was given an infusion of 25 I.U. of ACTH over 6 hours four weeks postoperatively. A normal rise in plasma 17-hydroxycorticosteroids occurred. We have no explanation for the lack of rise in his circulating steroids during surgery.

In the other patients there was a rise in the plasma 17-hydroxycorticosteroids during their operative procedures. There was a general tendency for the magnitude of the increase to be directly related to the duration of the procedure and its severity.

*Changes in plasma levels of 17-hydroxycorticosteroids following operative procedures*

The 17-hydroxycorticosteroid levels immediately following 33 operations and at two hourly intervals thereafter are shown in Table II. Figure 2 demonstrates the average levels after surgery; the changes following procedures lasting less than two hours are compared with those lasting more than two hours. Although operations lasting two hours or less were associated with a rise in 17-hydroxycorticosteroid plasma levels during the procedure, there was little change thereafter. Levels, however, did not return to the preoperative

TABLE II

*Effect of surgery on plasma concentrations of 17-hydroxycorticosteroids following operational procedures*

| No. | Age and sex | Length of op. *in hrs.* | Anesthesia | 17-OH corticosteroids (γ/*100 ml.*) in plasma after operation | | | | Operations and remarks |
|---|---|---|---|---|---|---|---|---|
| | | | | 0 hrs. | 2 hrs. | 4 hrs. | 6 hrs. | |
| 13 | 60 M | 2 | General | 21 | 20 | 19 | 18 | Removal of kidney stone |
| 14 | 65 M | 4 | High spinal | 23 | 46 | 84 | 102 | Gastrectomy |
| 15 | 60 M | 2 | High spinal | 14 | 22 | 20 | 20 | Prostatectomy |
| 16 | 40 M | 1½ | High spinal | 5 | 17 | 19 | 25 | Renal stone removed |
| 17 | 52 F | 4½ | General | 60 | | 48 | 24 | Cholecystectomy |
| 18 | 55 M | 4½ | High spinal | 20 | 40 | 68 | 100 | Cholecystectomy |
| 19 | 50 M | 4¾ | General | 34 | 35 | 45 | 29 | Bilateral thoraco-lumbar sympathectomy |
| 20 | 35 F | 4 | General | 15 | 21 | 38 | 31 | Cholecystectomy |
| 21 | 57 F | 4½ | General | 38 | 46 | 53 | 148 | Ca of pancreas. Jejuno-choledocostomy. Patient went into shock at end of op. Resp. poor |
| 22 | 30 F | 3 | General | 20 | 26 | 26 | 20 | Thyroidectomy |
| 23 | 38 M | 4 | General | 28 | 42 | 30 | 21 | Atrial appendectomy |
| 24 | 68 M | 1½ | Spinal | 10 | 10 | 17 | 19 | Bilateral sympathectomy for arteriosclerosis |
| 25 | 42 M | 3 | General | 19 | 21 | 17 | 35 | Thyroidectomy |
| 26a | 68 F | 1¼ | Pentothal and $NO_2$ | 18 | 10 | 10 | 20 | After skin incision pt. went into shock. Bleeding peptic ulcer. Operation stopped |
| 26b | 68 F | 5 | Pentothal and $NO_2$ | 46 | 55 | 66 | 40 | Gastric resection. BP low throughout the procedure |
| 27 | 72 M | 1 | Spinal | 31 | 26 | 24 | 20 | Suprapubic prostatectomy |
| 28 | 56 F | 3 | General | 41 | 35 | 34 | 18 | Excision of large abdominal cyst |
| 29 | 56 F | 2½ | Spinal and pentothal | 25 | 36 | 40 | 32 | Smith-Peterson nailing |
| 30 | 60 F | 4 | General | 19 | 27 | 38 | 40 | Cholecystectomy |
| 31 | 37 F | 2½ | General | 20 | 30 | 24 | 31 | Splenectomy |
| 32 | 57 F | 6¼ | General | 28 | 32 | 34 | 36 | Cholecystectomy |
| 33 | 55 M | 4 | Spinal | 12 | 19 | 22 | 23 | Open reduction and internal fixation of hip |
| 34 | 60 F | 5 | Spinal | 28 | 30 | 26 | 18 | Cholecystectomy. Patient jaundiced |
| 35 | 38 M | 2½ | Spinal | 6 | 16 | 28 | 35 | Intramedullary nailing of left femur |
| 36 | 60 M | 5 | Cyclo propa. and $NO_2$ | 13 | 22 | 47 | 33 | Cholecystectomy and duct exploration |
| 37 | 62 F | 7 | Pentothal and cyclo | 36 | 53 | 60 | 54 | Thyroidectomy |
| 38 | 62 F | 2½ | Spinal and cyclo | 29 | 25 | 29 | 28 | Bilateral sympathectomy |
| 39 | 60 F | 3 | Spinal | 25 | 40 | 35 | 15 | Right colectomy |
| 40 | 62 M | 4½ | General | 26 | | | | Gastrectomy |
| 41 | 25 F | 4½ | General | 42 | | | | Ligation of patent ductus |
| 42 | 60 M | 5½ | General | 34 | | | | Thyroidectomy (substernal) |
| 43 | 42 F | 3 | General | 30 | 36 | 34 | 34 | Removal of cyst of pericardium |
| 44 | 60 M | 4 | General | 30 | 33 | 40 | 20 | Removal of diverticulum of esophagus |

values during the period studied. The rise in 17-hydroxycorticosteroid levels both during and after operations lasting more than two hours was more striking.

The details of the operative procedures in a few of these patients may help to throw light on the significance of the steroid rises. One patient (No. 26) was a 68-year-old white female with auricular fibrillation and severe hemorrhage from a peptic ulcer. The patient had slight persistent bleeding from the ulcer at the time of surgery. On the first occasion, after she had been anesthesized with pentothal and nitrous oxide, her blood pressure fell moderately, immediately following superficial incision. The latter was closed and the patient was returned to the ward where she recovered uneventfully. The 17-hydroxycorticosteroids showed little change following the closure of the incision (lower curve in Figure 3). Without additional blood transfusion or other specific therapy the patient underwent a gastric resection the following day under general anesthesia. In spite of a similar hypotensive reaction an operation lasting five hours was completed successfully. As can be seen in Figure 3 (upper curve) there was a striking rise in 17-hydroxycorticosteroid levels

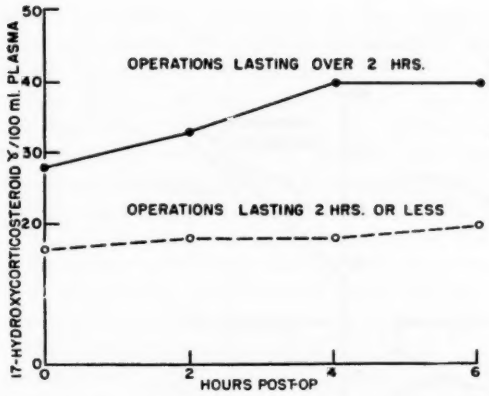


FIG. 2. AVERAGE PLASMA 17-HYDROXYCORTICOSTEROIDS FOLLOWING SURGERY

after the second operation. This case suggests that the magnitude of the rise in the 17-hydroxycorticosteroid levels in plasma is related to the severity of the surgical procedure and its duration.

In Figure 4 the changes in 17-hydroxycorticosteroids in a 57-year-old female following a cholecystectomy lasting four and one-half hours are shown. After the operation this patient's 17-hydroxycorticosteroid level reached 148 μg per 100 ml. of plasma. Such levels have not been observed in other subjects not undergoing surgery who were not moribund,[1] even following the continuous intravenous administration of maximally stimulating doses of ACTH over a period of 48 hours. This patient died 36 hours following the operation.

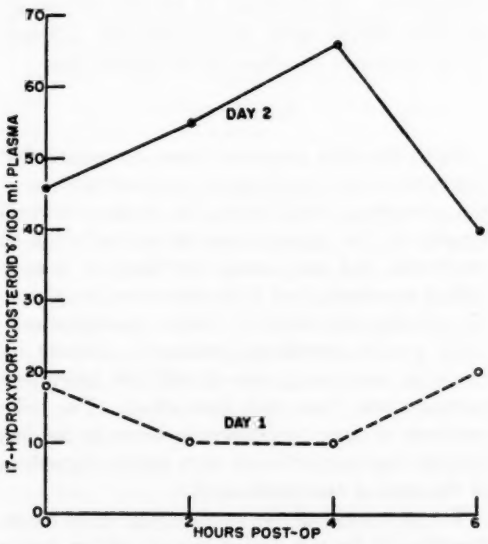


FIG. 3. PLASMA 17-HYDROXYCORTICOSTEROIDS IN A PATIENT IN WHOM ANESTHESIA WAS GIVEN AND ONLY A SUPERFICIAL INCISION WAS MADE ON ONE DAY (LOWER CURVE) AND A SUBTOTAL GASTRECTOMY THE FOLLOWING DAY (UPPER CURVE)

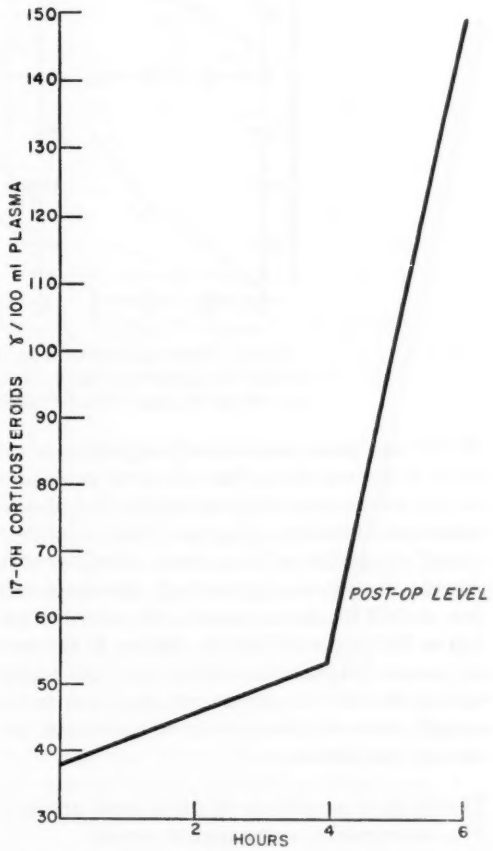


FIG. 4. PLASMA 17-HYDROXYCORTICOSTEROIDS IN A PATIENT FOLLOWING CHOLECYSTECTOMY LASTING FOUR AND ONE-HALF HOURS

### *ACTH response pre- and post-operatively*

Standard ACTH response tests were performed on six surgical patients at least one week before the operation. In each case a normal rise in 17-hydroxycorticosteroid levels occurred (Figure 5). After operation a similar rise was observed when

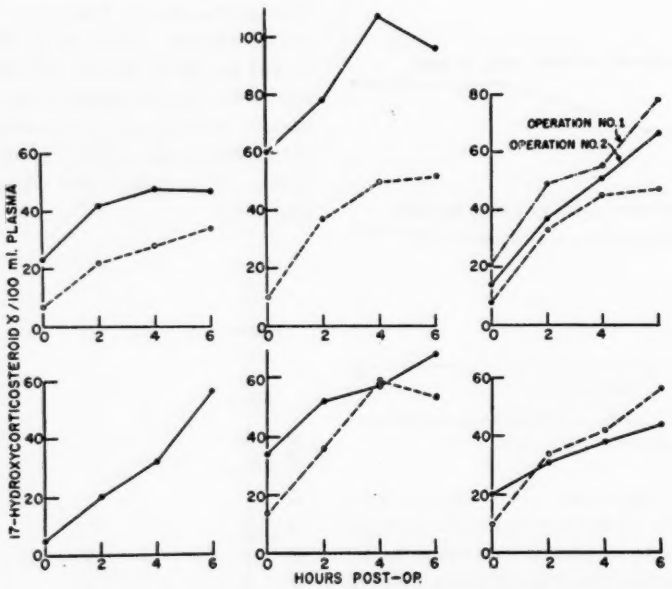


FIG. 5. RESPONSE OF PLASMA 17-HYDROXYCORTICOSTEROIDS TO 25 I.U. ACTH INTRAVENOUSLY ON A CONTROL DAY (BROKEN LINES) AND FOLLOWING MAJOR SURGERY (SOLID LINES)

ACTH was given intravenously regardless of the levels of the steroids at the end of the procedure. In one of these cases the postoperative 17-hydroxycorticosteroid level was 60 μg per 100 ml. of plasma, a level compatible with maximal adrenal cortical stimulation. Interestingly enough, the administration of ACTH was associated with a further rise (up to 107 μg per 100 ml. of plasma) in the level of plasma 17-hydroxycorticosteroids; the magnitude of the rise was as great as that seen in the control curve in this patient or in normal unstressed individuals.

*The effects of surgery on the blood levels produced by intravenously administered steroids*

Each of five surgical patients was given hydrocortisone intravenously on two occasions. A control injection was given at least one week before or four weeks after the operation. The same patients received hydrocortisone during or following their operation. On each occasion the plasma 17-hydroxycorticosteroid levels were measured for a period of 8 hours (Figure 6). In three patients (G, H and I) the levels of 17-hydroxycorticosteroids reached higher values and were maintained at higher levels after surgery than during the control periods. In two cases (J and K), the disappearance curves were not significantly different on the operative day than on the control day.

## DISCUSSION

From the data presented here it appears that there are at least two factors associated with surgical procedures which affect the levels of adrenal steroids in the general circulation; induction of anesthesia and the actual handling of tissues. Spinal anesthesia had little effect on the plasma 17-hydroxycorticosteroid levels preoperatively, while general anesthesia produced a definite increase in most cases, even though the subsequent operation may have had little effect. The independence of these two effects is shown by the fact that the post-surgical rises were similar regardless of the type of anesthesia used.

The post-surgical rise would appear to be a continuation of the rise during surgery rather than a separate phenomenon. Both were correlated with the length of operation and the extent of tissue trauma. The post-operative rise probably is the result of the continued presence of damaged tis-

sues. Epinephrine apparently is not involved since the plasma 17-hydroxycorticosteroids fail to rise following its administration (9).

The adrenals of these patients were certainly not maximally stimulated, since the administration of ACTH was associated with an additional rise above the high levels of 17-hydroxycorticosteroids induced by the surgical procedures. It is of interest that in a few patients the rate of rise of the steroid level, either when ACTH was given or in its absence, exceeded that which we have observed in surgical patients or in normal individuals receiving amounts of ACTH which produce maximal rises in plasma 17-hydroxycorticosteroid levels under basal conditions. Thus, the data cannot be explained entirely by endogenous ACTH secretion. It must, therefore, be concluded that factors other than adrenal cortical stimulation play a part in raising the steroid concentrations during and subsequent to surgery. It follows that the removal of adrenal steroids from the circulation may be impaired. That this is a significant factor is suggested by the decreased rate of disappearance of injected hydrocortisone in three of the five patients in whom this type of study was performed during or immediately after surgery. There is evidence to suggest that the liver has an important role in these processes. Data concerning the importance of the liver in the metabolism of adrenal steroids is being presented in other papers of this series (18, 19).

It should be emphasized, however, that these data do not exclude the possibility that the adrenal cortex is stimulated submaximally by the surgical trauma. Indeed, if the spontaneous post-surgical rises in plasma 17-hydroxycorticosteroid level were entirely due to impaired extra-adrenal metabolism and excretion of these compounds, we should have observed supramaximal rises regularly when ACTH was administered in the presence of an already elevated level. The rate of disappearance of the additional steroid secreted by the stimulated adrenal would also presumably have been impaired, resulting in increments of considerably greater magnitude than those observed under basal circumstances. This was clearly not the case. Similarly *each* patient receiving exogenous steroid at a time when his level was elevated should have shown markedly impaired removal of the steroid if the elevation

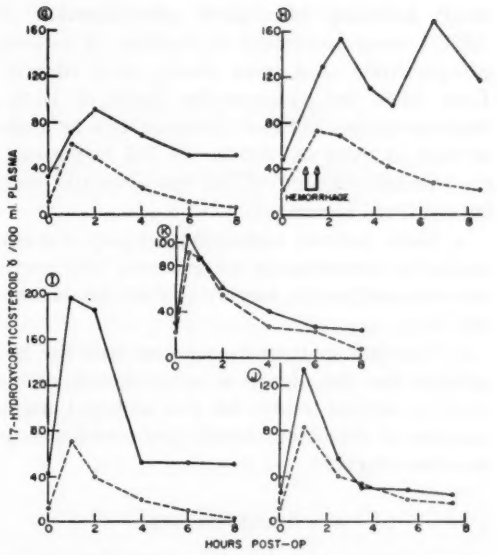


FIG. 6. PLASMA 17-HYDROXYCORTICOSTEROIDS FOLLOWING INTRAVENOUS ADMINISTRATION OF 50 MG. OF HYDROCORTISONE ON A CONTROL DAY (BROKEN LINE) AND FOLLOWING SURGERY (SOLID LINES)

resulted only from a decreased rate of removal. Thus these data suggest a combined effect of increased production and decreased removal.

Although the increased plasma 17-hydroxycorticosteroids in the post-surgical period probably are metabolically active, whether the higher levels are due to increased adrenal secretion or impaired disposition, for the reasons considered in the introduction, our finding that surgical trauma is associated with an increase in the levels of circulating adrenal steroids does not necessarily indicate that the entire adrenal-like metabolic response to surgery is mediated by these rises.

## SUMMARY

1. The plasma levels of 17-hydroxycorticosteroids were determined in a group of surgical patients. They were found to be elevated following the induction of anesthesia and during and after surgery. Following surgery the increases were roughly proportional to the duration and severity of the operative procedure. This elevation usually persisted at least 6 hours following surgery.

2. In an attempt to measure adrenal cortical capacity, the changes in 17-hydroxycorticosteroid

levels following intravenous administration of ACTH were determined in a group of patients preoperatively, and again shortly after surgery. Even when the post-operative levels of 17-hydroxycorticosteroids rose spontaneously to levels as high as those induced by ACTH in normals, the administration of ACTH was associated with an additional increment.

3. Some patients undergoing surgery did not metabolize intravenously administered hydrocortisone as rapidly after surgery as they did on control days.

4. The data seem most consistent with the hypothesis that the adrenal is submaximally stimulated by surgical trauma but that impaired disappearance of circulating steroid also contributes to the rises observed.

ACKNOWLEDGMENTS

We are grateful to Dr. E. M. Alpert of Merck and Company for generous supplies of steroids and to Dr. E. E. Hays of Armour and Company for generous supplies of ACTH. We wish to extend special thanks to the members of the Department of Surgery and Anesthesia for their whole hearted cooperation in making this study possible.

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# THE ROLE OF THE LIVER AND THE ADRENAL IN PRODUCING ELEVATED PLASMA 17-HYDROXYCORTICOSTEROID LEVELS IN SURGERY[1]

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(Submitted for publication April 7, 1954; accepted July 15, 1954)

In another paper of this series (1) it was demonstrated that most patients undergoing surgery developed increased plasma levels of 17-hydroxycorticosteroids. Evidence was presented that these rises are mediated in part by extra-adrenal factors. The concept that extra-adrenal factors may affect adrenal steroid levels is not a new one but little evidence to support such a concept has been presented.

The liver is known to contain enzyme systems capable of modifying these compounds *in vitro* (2). The reduced effect of the diseased liver in estrogen metabolism has been advanced by many authors as the explanation of the clinical evidence suggestive of estrogenic overactivity in patients with cirrhosis of the liver (3). That liver disease results in profound alteration of adrenocortical steroid metabolism also, has been demonstrated in current studies by Brown, Willardson, Samuels, and Tyler (4).

Thus it seemed logical to study the relation of hepatic function to the magnitude of the rise in 17-hydroxycorticosteroids after surgery. In addition, it was hoped that by the determination of free and conjugated steroids of this type in the urine an estimate of the total adrenal output of these substances would be obtained. By the correlation of the data it was expected that the influence of increased adrenal secretion and of impaired steriod disappearance resulting from hepatic dysfunction could be evaluated at least on a qualitative basis. In this report data dealing with these aspects of the effect of surgery on adrenal steroid metabolism will be presented and an attempt made to relate this information to the overall picture of the regulation of adrenal cortical secretion, insofar as this is possible on the basis of the evidence now available.

## MATERIALS AND METHODS

Twenty relatively healthy patients who were to undergo elective surgery were selected so that the series would include those having major and minor procedures, both sexes and various ages. During the preoperative period a sulfobromphthalein (BSP) excretion curve was determined on each patient. Five mg. per kilo of BSP dye was injected intravenously within one minute. Blood was drawn before the dye injection and at 10- to 12-minute intervals afterward until four additional samples had been collected. The time of each sample was carefully noted. The serum BSP concentration was determined as mg. per 100 ml. of serum by a modification of the Rosenthal method (5). (The usual clinical expression of dye concentration is in terms of per cent retention, which assumes that an injection of 5 mg. per kilo will produce a serum level of 10 mg. per 100 ml. arbitrarily taken as 100 per cent.) Analysis of these data was made by plotting the dye concentration as the ordinate on a logarithmic scale against time on the abscissa. In normal individuals straight lines were obtained by this technique and the rate of clearance could be calculated easily. From the graphs accurate estimation of the 45-minute retention could also be made. Between the first and second hour after surgery a second BSP excretion study was performed in the same fashion on each patient. Many of these curves differed from the control observations in that straight lines were not obtained when the data were plotted. The initial clearance rates were frequently more rapid than those found during the latter half of the 45-minute period. As a result, valid expression of the data as clearance rates was not possible. They are therefore recorded here in the usual clinical fashion as per cent dye retention at 45 minutes.

Heparinized plasma for 17-hydroxycorticosteroid determination was obtained from each patient at 8 A.M. on one or more days pre-operatively. Similar samples were drawn at two, four, six, and eight hours post-operatively on each patient. Plasma 17-hydroxycorticos-

[1] This investigation was supported in part by research grants from the National Institutes of Health, U. S. Public Health Service and from Armour and Co. and in part by a contract (DA-49-007MD-136) between the University of Utah and the Department of the Army.

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teroids were determined by a modification of the method of Eik-Nes, Nelson, and Samuels (6).

In eight representative patients of the group, 24-hour urine collections (7 A.M. to 7 A.M.) were made on a control day and on the day of the operation. The creatinine content of each sample was determined to indicate the completeness of the collection. In each sample the free and total 17-hydroxycorticosteroids were estimated by a technique previously described (4).

## RESULTS

In Table I the patients have been listed in descending order of the highest steroid level observed post-operatively. The sex and age of each patient, the nature and duration of the operation, the anesthesia used, the post-operative condition and BSP retention pre- and post-operatively, are recorded as well as the steroid levels observed. The average control 17-hydroxycorticosteroid level of 11.7 μg per 100 ml. of plasma, was similar to that found in normal individuals (7). It will be noted that the increase in BSP retention post-operatively was higher in those patients who showed the higher steroid rises. This is illustrated graphically in Figure 1. Both values also show some correlation with the duration of the operation.

Patients Nos. 5 and 14 showed considerably more BSP retention in relation to the steroid rise observed than the remainder of the group. Patient No. 14 had failed to excrete BSP normally before operation; very probably the large post-operative increase is not as significant as an index of the degree of additional liver dysfunction as a similar increase in patients with initially normal liver function would be. It may be important that both of these patients had hypertensive heart disease and minimal congestive heart failure at the time of operation.

In six of the twenty patients in this study the plasma 17-hydroxycorticosteroids did not rise above 23 μg per 100 ml. of plasma, which represents the approximate upper limit of normal values at 8 A.M. However, because of the characteristic diurnal curve of these substances and the fact that the postoperative values extended into the afternoon, only the last two patients can be said to have failed to show a significant elevation of the steroid level. None of these six patients showed a significant increase in BSP retention post-operatively. In three additional patients (Nos. 9, 11, and 13) only a small increase in BSP retention was observed post-operatively and even these values were within the usually accepted normal range. All three showed moderate increases in 17-hydroxycorticosteroid levels. Patient No.

TABLE I

*The effects of surgery on BSP retention and plasma levels of 17-hydroxycorticosteroids in twenty patients*

| Pt. No. | Sex and age | Operation | Duration (*hrs.*) | Anesthetic | Condition post-op. | % BSP retention at 45′ | | | 17-OH steroid plasma level | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Pre-op. | Post-op. | Diff. | Controls average | (γ/*100 ml.*) 2 | 4 | 6 | 8 |
| 1 | F 60 | Cholecystectomy | 3 | Ether | Shock | 7 | 32 | 25 | 8 | 31 | 40 | 60 | 100 |
| 2 | M 75 | Herniorrhaphy | 2 | Spinal | Good | 6 | 18 | 12 | 20 | 100 | 52 | 62 | 66 |
| *3 | M 61 | Gastric resection | 3½ | Cyclo | Good | 0 | 18 | 18 | 12 | 39 | 40 | 53 | 26 |
| 4 | M 78 | Perineal prostatectomy | 1¼ | Spinal | Good | 0 | 29 | 29 | 13 | 31 | 45 | 52 | 52 |
| *5 | F 68 | Bilat. vein ligation | 1½ | Spinal | Ht. failure | 10 | 50 | 40 | 13 | 45 | 25 | 21 | 19 |
| *6 | M 69 | Herniorrhaphy | 1¾ | Spinal | Good | 10 | 20 | 10 | 10 | 30 | 44 | 40 | 40 |
| *7 | M 53 | Gastric resection | 3½ | Cyclo | Good | 3 | 21 | 18 | 11 | 34 | 39 | 44 | 30 |
| 8 | M 47 | Bilat. vein ligation | 1¾ | Spinal | Good | 10 | 23 | 13 | 12 | 37 | 32 | 14 | 17 |
| 9 | M 25 | Submucous resection | 2 | Local | Good | 2 | 8 | 6 | 17 | 25 | 24 | 33 | † |
| 10 | F 51 | Submucous resection | 1 | Local | Good | 16 | 26 | 10 | 9 | 9 | 31 | 12 | 10 |
| 11 | M 49 | Fusion of knee | 1¾ | Cyclo | Shock | 0 | 5 | 5 | 12 | 25 | 28 | 27 | 31 |
| 12 | M 77 | Suprapubic prostatectomy | 1½ | Spinal | Good | 2 | 16 | 14 | 9 | 30 | 26 | 26 | 19 |
| *13 | F 36 | Tibial bone graft | 2 | Spinal | Good | 3 | 9 | 6 | 14 | 22 | 12 | 27 | 19 |
| *14 | F 55 | Pin removal | 1½ | Cyclo | Lryngl. sp. ht. failure | 30 | 51 | 21 | 12 | 20 | 21 | 27 | 27 |
| 15 | M 47 | Sequestrectomy | 1½ | Spinal | Good | 1 | 1 | 0 | 9 | 12 | 20 | 20 | 23 |
| 16 | M 28 | Tendonotomy | 1½ | Pento | Good | 5 | 8 | 3 | 18 | 7 | 10 | 20 | 20 |
| 17 | M 40 | Resection of toe | 1 | Local | Good | 10 | 12 | 2 | 15 | 19 | 16 | 19 | 16 |
| *18 | F 20 | Herniorrhaphy | 1½ | Spinal | Good | 4 | 4 | 0 | 23 | 18 | † | 16 | 16 |
| *19 | M 49 | Hemorrhoidectomy | ¾ | Caudal | Good | 2 | 4 | 2 | 9 | 3 | 6 | 8 | 7 |
| 20 | M 14 | Vasectomy | 1 | Local | Good | 5 | 5 | 0 | 11 | 7 | 7 | 7 | 7 |

* Pre- and post-operative studies of urinary 17-hydroxycorticosteroids.
† Samples lost.

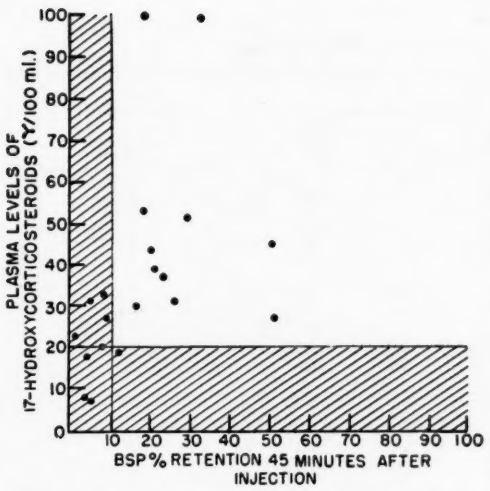


FIG. 1. RELATION OF POST-OPERATIVE INCREASE IN BSP RETENTION AT 45 MINUTES TO THE MAXIMAL PLASMA 17-HYDROXYCORTICOSTEROID LEVEL OBSERVED DURING THIS PERIOD

11 developed mild shock after the post-operative BSP determination had been completed and patient No. 9 was emotionally disturbed and excited to an unusual degree during the first few hours following his submucous resection.

The character of the data obtained is demonstrated more clearly in Figure 2. Here the findings in two representative patients (No. 3 and No. 19) are recorded in detail. They show the correlation of impaired BSP excretion and the magnitude of the post-operative 17-hydroxycorticosteroid rise.

The urinary excretion of free and conjugated 17-hydroxycorticosteroids by the eight patients in whom the observations were made is recorded in Table II. Again they are listed in the order of maximal plasma steroid level observed post-operatively. The control values fell within the range of values which have been observed in normal individuals (4). During the day of operation a rise in steroid excretion was observed in each patient except No. 19. He also failed to show a change in BSP excretion or a significant rise in plasma steroid levels post-operatively. While the others showed significant increases in urinary 17-hydroxycorticosteroid excretion, there was no obvious correlation between the magnitude of that rise and the degree of change in BSP clearance or plasma steroid level.

The excretion of free 17-hydroxycorticosteroids was only a small fraction of the total output. However, with the exception of patient No. 14, the relative increase in this fraction was larger than the increase in the total excretion of steroids. This would either indicate that the relative efficiency of conjugation was reduced or that renal clearances of the free and conjugated compounds are dissimilar.

## DISCUSSION

We have presented evidence that the magnitude of the rise in plasma 17-hydroxycorticosteroids following surgery shows some correlation with the increase in BSP retention occasioned by this trauma and with the duration and severity of the surgical procedure. Although the total, free, and conjugated 17-hydroxycorticosteroids in the urine showed an increase in all but one of the eight patients in whom they were determined, their

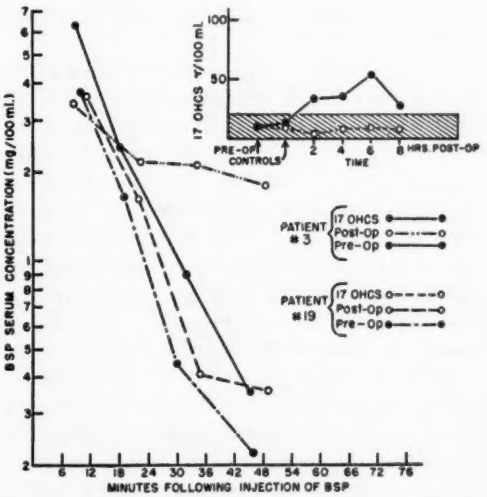


FIG. 2. DETAILED BSP AND 17-HYDROXYCORTICOSTEROID (17-OHCS) CURVES OBSERVED IN TWO REPRESENTATIVE PATIENTS

Note that no elevation of steroid level occurred post-operatively in patient No. 19 and that he excreted BSP essentially as well post-operatively as pre-operatively. In contrast, patient No. 3 had a marked rise in 17-OHCS levels and showed impairment of BSP excretion post-operatively.

TABLE II

*Urinary 17-hydroxycorticosteroids in eight patients undergoing surgery*

| Patient No. | Collection | 17-Hydroxycorticosteroids | | | Maximal plasma level 17-hydroxycorticosteroids |
|---|---|---|---|---|---|
| | | Free mg./24 hr. | Conj. mg./24 hr. | Total mg./24 hr. | γ/100 ml. |
| 3 | Pre-op. | .15 | 2.1 | 2.25 | 13 |
| | *Post-op. | .99 | 8.0 | 8.99 | 53 |
| 5 | Pre-op. | .17 | 4.5 | 4.67 | 13 |
| | *Post-op. | .52 | 5.6 | 6.12 | 45 |
| 6 | Pre-op. | .18 | 2.4 | 2.58 | 11 |
| | *Post-op. | .71 | 8.9 | 9.61 | 44 |
| 7 | Pre-op. | .15 | 2.0 | 2.15 | 12 |
| | Post-op. | 1.5 | 6.4 | 7.9 | 39 |
| 13 | Pre-op. | .16 | 4.9 | 5.06 | 17 |
| | Post-op. | .87 | 10.8 | 11.67 | 27 |
| 14 | Pre-op. | .40 | 5.6 | 6.0 | 12 |
| | Post-op. | .43 | 9.1 | 9.53 | 27 |
| 18 | Pre-op. | .11 | 4.4 | 4.51 | 23 |
| | Post-op. | .43 | 8.0 | 8.43 | 18 |
| 19 | Pre-op. | .25 | 5.6 | 5.85 | 10 |
| | Post-op. | .36 | 4.8 | 5.16 | 8 |

* Collection incomplete.

magnitudes seemed to have no uniform relation to the other factors.

Certain limitations in the interpretation of our observations should be emphasized. First, the relation of the change in BSP excretion to the rise in plasma levels of adrenocortical steroids offers only presumptive evidence that functional impairment of the liver plays a role in the observed steroid changes. Second, the BSP clearance measures two factors: the rate of hepatic blood flow and the ability of the liver cells to excrete the dye. There is some evidence to suggest that the excretory function of the liver is a biphasic phenomenon in which the dye is first concentrated, possibly by the Kuppfer cells, and then excreted in the bile by an additional mechanism (8). The biphasic character of the post-operative excretion patterns which we have observed would seem to confirm this hypothesis. In addition, impaired ability of the liver to excrete BSP, even though that process involves multiple mechanisms, probably involves different systems than those concerned in the removal of corticosteroids from the circulation. Nevertheless, some evidence of a relation between these mechanisms in the usual clinical varieties of liver disease has been demonstrated by Brown, Willardson, Samuels, and Tyler (4). It is possible that there is a similar relationship in the more acute hepatic impairment which was studied here.

It may be added that a number of previous observers have pointed out that a decrease in liver function ocurs during and following anesthesia (9) and/or surgical procedures (10–12). They have shown that the severity of the trauma is related to the degree of liver impairment. This tends to confirm the significance of the changes observed in our own relatively small series.

A third limitation is concerned with the time relationships involved. For technical reasons plasma 17-hydroxycorticosteroids were determined at intervals over an eight-hour period while BSP clearance was studied during only a 45-minute interval in that period. Thus if, as seems probable, very rapid changes are occurring in hepatic function the BSP value obtained may not represent the average situation during the eight-hour period. Another discrepancy in time is the use of 24-hour urine collections, which was dictated not only by clinical convenience but also because of the delay in the excretion of steroids and the

marked diurnal variation in their rate of excretion under basal conditions (13).

In spite of these limitations the correlations seem evident. Because of these limitations, however, more significance must be given in the interpretation of the data to the qualitative character of the changes than to their actual magnitude.

Thorn, Jenkins, Laidlaw, Goetz, and Reddy (14) have reported that the total urinary adrenal corticosteroids are increased after major operative procedures but they did not examine the free and conjugated fractions in their studies. The magnitudes of the changes which they observed are similar to those reported here. They also observed that minor operative procedures and relatively mild environmental "stresses" did not produce an increase in the urinary excretion of total 17-hydroxycorticosteroids. Their observations, as well as those presented here, would seem to confirm our previous conclusion (1) that increased adrenal secretion contributes to the elevation of the plasma steroid level during and following major surgical procedures.

A review of the factors which are now known, or are thought, to be concerned in the metabolism of adrenal steroids may help in understanding the significance of the observations presented here and should point out the areas in which problems remain.

As is diagrammatically represented in Figure 3, a number of factors are concerned in the regulation of circulating adrenal cortical steroid levels. On the positive side the secretion of the adrenal cortex is apparently of primary importance. As we have demonstrated (15), the peripheral blood level depends, at least in part, on the rate at which ACTH reaches the gland. There is, however, a maximal response which cannot be exceeded by increasing the concentration of ACTH. This level probably is determined by the maximum rate of sustained secretion by the adrenal gland.

As we go clockwise about the diagram the next factor is related to the tissues other than the liver and kidney. At present it is impossible to measure the amounts of adrenal steroids in these tissues, their rate of exchange or the speed of their destruction. Previous attempts to demonstrate tissue removal of 17-hydroxycorticosteroids by studies of arteriovenous differences across the femoral

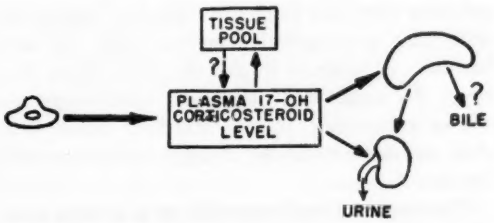


FIG. 3. DIAGRAMMATIC REPRESENTATION OF THE FACTORS AFFECTING PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS

circulation both in normal and in inflamed extremities have been unsuccessful (16). The rate of removal and of alteration of the steroids in the tissues must, therefore, presumably be less than the limits of measurement available for such a study at present.

On the other hand, current and previous studies in our laboratory have demonstrated a significant decrease in plasma levels across the hepatic circulation (17). Thus it would appear that the liver is the main site of removal of the 17-hydroxycorticosteroids which are measured by the procedure used in these studies. Renal excretion of steroids in the free form is extremely small except when the plasma levels are greatly elevated. Moderate amounts appear in conjugated form after oral or parenteral administration. This fraction never exceeds approximately one-third of the administered steroid and is usually very much less than this (13). These conjugates presumably are formed in the liver and are not measured by our plasma method.

These considerations indicate that the main factor concerned in the regulation of plasma levels, other than adrenal secretion, is the liver. The correlations which are presented in this report are consistent with the hypothesis that hepatic removal of steroids from the circulation is an important factor in determining the peripheral plasma level. As discussed above, the techniques which we have used are not adequate to distinguish between changes in blood flow and functional damage to the liver cells but the persistent impairment of BSP excretion for a period of days post-operatively, which has been shown by others, suggests that the latter occurs. Evidence of impaired liver blood flow has also been presented and it seems

probable that both factors are involved during the immediate post-operative period which we have studied. It seems to us that the effect of the liver must be considered whenever conclusions are drawn concerning adrenal secretion either from data based on metabolic changes or from steroid levels.

Persistent adrenal secretion at a greater-than-normal rate presumably resulting from endogenous ACTH, at a time when the steroid level is already elevated would seem to argue against the hypothesis presented by Sayers and Sayers (18) that the steroid level regulates the output of ACTH. The observed diurnal variation in normal men and the rather wide variability of normal levels at any given time also have been interpreted as evidence against a homeostatic mechanism (7). However, the consistency of the average value in normals, in patients with liver disease where impaired removal has been demonstrated (4), and in a wide variety of chronic illnesses (19) would seem to indicate that there is a homeostatic mechanism which responds less rapidly than that controlling certain other substances, such as the blood sugar level, but none-the-less functions quite effectively over longer intervals. In addition, there must be a mechanism which calls forth increased secretion acutely in the presence of severe trauma such as a major surgical procedure.

## SUMMARY

1. After surgical operations the magnitude of the plasma 17-hydroxycorticosteroid levels attained shows a correlation with impaired BSP excretion and the length and severity of the operative procedure.

2. Conjugated urinary 17-hydroxycorticosteroids increased after all except one brief, minor procedure but showed no correlation with the plasma levels attained.

3. The factors which affect the plasma levels are discussed.

4. The hypothesis is proposed that the increased plasma levels of 17-hydroxycorticosteroid after surgery are the result of both increased adrenal secretion of these steroids and impaired hepatic removal.

## ACKNOWLEDGMENTS

We are grateful to Dr. E. E. Hays of Armour and Company for generous supplies of ACTH, and to Smith, Kline and French for part of the BSP used. We wish to extend special thanks to the members of the Departments of Surgery and Anesthesia for their wholehearted cooperation in making this study possible.

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# 17–HYDROXYCORTICOSTEROID METABOLISM IN LIVER DISEASE [1]

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(Submitted for publication April 7, 1954; accepted July 15, 1954)

Considerable evidence has been accumulated to indicate that the liver is concerned in the metabolism of steroids (1). The development of methods for the measurement of 17-hydroxycorticosteroids in the plasma (2) and in the urine (3) has enabled us to study the role of the liver in the handling of 17-hydroxycorticosterone (hydrocortisone) and related compounds. This communication will consider the plasma levels and the urinary excretion of the 17-hydroxycorticoids in patients with liver disease as compared with a group of normal subjects.

## MATERIALS AND METHODS

Twelve patients with liver disease and eleven normal subjects were studied. Of the twelve patients with liver disease, there were nine with cirrhosis, two with hepatitis and one with metastatic carcinoma. Serial studies were carried out in the patients with hepatitis to note the effects of improvement in liver function. The "normal subjects" were hospital personnel or convalescing hospital patients who were free of liver disease, fever, and endocrine abnormalities.

These subjects were given intravenously a solution containing 0.02 per cent hydrocortisone (free alcohol), 5 per cent dextrose and 1 per cent alcohol. A sufficient quantity of this commercially-prepared solution [2] was administered to give the subject 1 mg. of hydrocortisone for each kilogram of body weight.

Control bloods were drawn shortly after 8 A.M., following which the infusion was administered over a 30-minute period. Blood samples for the estimation of the 17-hydroxycorticosteroid level of the plasma were drawn at 1, 2, 4, and 6 hours after the beginning of the infusion. After the infusion, the subjects were allowed to have breakfast and to be up and about, if they were ambulatory.

The plasma was separated within two hours and stored in a frozen state until the analysis could be carried out. The estimation of the plasma 17-hydroxycorticosteroid level was performed in accordance with the modified technique of Nelson, Samuels, and Eik-Nes (2, 4).

Urines were collected for 24-hour periods and stored in the refrigerator without a preservative. In several studies, the urine samples on the day of the hydrocortisone were divided into 2-hour collections for the first six hours and then into 6-hour periods. After the collection period, an aliquot was stored in the frozen state until subsequent analysis for creatinine (5) and for free and conjugated 17-hydroxycorticoids.

The 17-hydroxycorticoid level in the urine was measured by a modification (6) of the technique of Reddy, Jenkins, and Thorn (3). Chloroform prepared as described by Eik-Nes, Nelson, and Samuels (4) was used for the extraction of the unconjugated compounds from urine. The Porter-Silber reaction was carried out on this residue as described by Reddy, Jenkins, and Thorn (3) and the results were reported in terms of a cortisone standard.

The total urinary 17-hydroxycorticoids were determined on a butanol extract of urine as described by Reddy, Jenkins, and Thorn (3) except for the following modification.

Because of the high optical density values obtained in the reagent blanks involving butanol, the n-butyl alcohol was treated in the following manner: 700 ml. of n-butyl alcohol (Merck) was put in a 1-liter distilling flask and the pH was adjusted to 1–2 with 50 per cent $H_2SO_4$ (v./v.). About 50 to 60 mg. of phenylhydrazine, which had been twice recrystallized from absolute alcohol, was added and the mixture was allowed to react at 60° C. for thirty minutes and then distilled. The first 50 ml. of distillate was discarded and distillation of the solution was continued until only about 100 ml. of a dark brown residue remained in the distilling flask. The redistilled n-butyl alcohol was stored in a dark brown bottle in the refrigerator and was stable for at least two to three weeks. This procedure removes nearly all of the interfering chromogenic material from the butanol, as can be seen in Table I. The values for conjugated 17-hydroxycorticoid in urine were estimated by subtraction of the value for the free from the total 17-hydroxycorticoid value.

Liver function was evaluated in each subject by the usual liver function tests, but particular attention was paid to the sulfobromphthalein (BSP) retention at 45 minutes, using 5 mg. per Kg. of body weight (7).

In eight subjects with cirrhosis of the liver, 25 In-

[1] Part of this study was presented at the meeting of the American Society for Clinical Investigation at Atlantic City, N. J., May 4, 1954.

[2] Generously supplied by Merck and Company.

TABLE I

*Effect of treatment of N-butyl alcohol on the optical density of reagent blanks* *

| | Tube "A" | Tube "B" | C.O.D. "A"—"B" | C.O.D. of reagent blank Range of 12 specimens |
|---|---|---|---|---|
| Untreated | .100 | .040 | .060 | .055–.090 |
| N-butyl alcohol | .115 | .035 | .080 | |
| Treated | .015 | .010 | .005 | .004–.010 |
| N-butyl alcohol | .030 | .020 | .010 | |

* Tube "B" contained n-butyl alcohol plus $H_2SO_4$ (620 ml. concentrated $H_2SO_4$ diluted to one liter with distilled water). Tube "A" contained n-butyl alcohol plus the phenylhydrazine-$H_2SO_4$ mixture. The optical densities were read at 410λ on a Coleman Junior spectrophotometer. The optical density reading of Tube "A" minus the reading of Tube "B" gave a corrected optical density (C.O.D.) value, which was due to the phenylhydrazine and any reaction of the phenylhydrazine with products in the n-butyl alcohol which might cause the formation of chromogens. The results in two instances are tabulated. In the last column, the range is given for twelve specimens.

ternational Units (I.U.) of adrenocorticotrophic hormone were administered intravenously over a 6-hour period and the plasma levels of 17-hydroxycorticosteroids were measured at 2, 4, 6, 8, and 10 hours after the beginning of the infusion (8).

Two normal subjects and two patients with severe cirrhosis of the liver were given an infusion of 1 mg. per Kg. of tetrahydrocortisone. This material was first dissolved in a small volume of absolute alcohol (50 mg. per ml.), the solution being then added to 250 ml. of normal saline. This was administered intravenously over a 30-minute period and the bloods were drawn at 30, 45, 60, 90 and 120 minutes after the beginning of the infusion for the determination of the plasma 17-hydroxycorticosteroid level.

## RESULTS

### *Plasma 17-hydroxycorticosteroid levels after the infusion of hydrocortisone*

The plasma levels of 17-hydroxycorticosteroids after the infusion of hydrocortisone are shown in Table II. It is to be noted that the plasma levels of the 17-hydroxycorticosteroids were not significantly different in the patients with liver disease from those of the normal subjects prior to the infusion. However, in the patients with liver disease, the levels were significantly higher after the standard infusion. In Figure 1, the plasma level expressed logarithmically is plotted against time. In both groups of cases, the rate of removal was proportional to the concentration at any given time, but the 17-hydroxycorticosteroids disappeared more slowly in the liver disease group.

At one hour after the beginning of the infusion, less than 5 per cent of the infused steroid could be accounted for in the plasma by multiplying an estimated plasma volume by the 17-hydroxycorticosteroid plasma level. This was noted even when the infusion lasted 59 minutes.

Two patients with homologous serum hepatitis were given hydrocortisone infusions on two sepa-

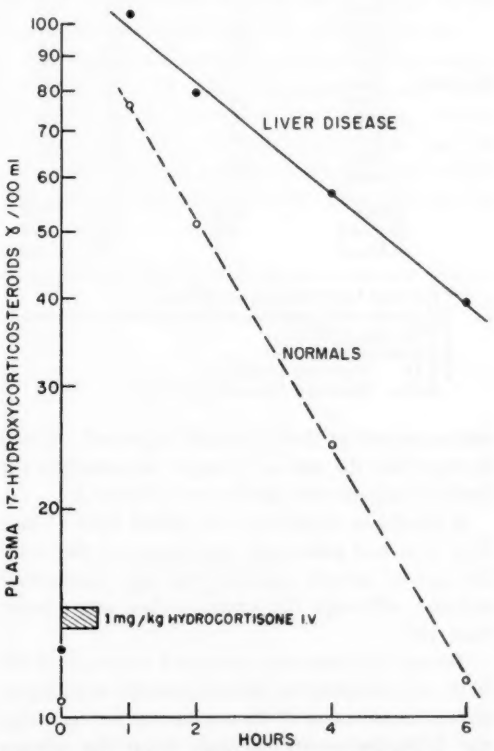


FIG. 1. THE MEAN 17-HYDROXYCORTICOSTEROID LEVELS IN THE PLASMA AFTER A STANDARD INTRAVENOUS INFUSION OF HYDROCORTISONE 1 MG. PER KG. ADMINISTERED OVER 30 MINUTES

The data are given in Table II.

TABLE II

*Effect of hydrocortisone infusion on plasma 17-hydroxycorticosteroid levels*

| | Patient | Control | Hours 1 | 2 | 4 | 6 | Bromsulphalein retention at 45 min. | Diagnosis |
|---|---|---|---|---|---|---|---|---|
| | | | γ/100 ml. | | | | % | |
| | Cor*† | 14 | 81 | 61 | 38 | 21 | 26 | Infectious hepatitis |
| | Mee‡ | 8 | 79 | 65 | 44 | 31 | 7 | Cirrhosis |
| | Pur† | 12 | 124 | 105 | 85 | 70 | 70 | Homologous serum hepatitis |
| | Wad* | 18 | 151 | 117 | 93 | 59 | 36 | Cirrhosis |
| | Phi | 11 | 103 | 99 | 68 | 56 | 48 | Cirrhosis |
| | Jep* | 15 | 127 | 91 | 86 | 60 | 32 | Cirrhosis |
| Liver | Sie | 5 | 66 | 45 | 25 | 7 | 16 | Cirrhosis |
| disease | Tur‡ | 12 | 110 | 82 | 53 | 42 | 16 | Cirrhosis |
| | War§ | 17 | 81 | 46 | 41 | 37 | | Metastatic cancer of liver |
| | Mee‡ | 10 | 84 | 63 | 42 | 25 | 7 | Cirrhosis |
| | Wad* | 19 | 129 | 107 | 68 | 41 | 27 | Cirrhosis |
| | Pur† | 16 | 112 | 82 | 56 | 30 | 21 | Homologous serum he patitis |
| | Qui | 8 | 87 | 63 | 42 | 27 | 9 | |
| | Phi | 11 | 113 | 81 | 53 | 41 | 35 | |
| | Mean | 12.6 | 103.4 | 79.1 | 56.7 | 39.1 | | |
| | S.D.± | 4.0 | 23.5 | 22.0 | 19.8 | 16.8 | | |
| | $S.D._m$± | 1.1 | 6.5 | 6.1 | 5.5 | 4.7 | | |
| | Sin‡ | 7 | 56 | 43 | 16 | 6 | 2 | Normal |
| | Sim‡ | 9 | 64 | 45 | 23 | 11 | 3 | Normal |
| | And‡ | 14 | 71 | 55 | 20 | 11 | 3 | Normal |
| | Sha | 15 | 56 | 48 | 23 | 12 | 5 | Normal |
| | Car | 8 | 100 | 50 | 28 | 16 | 3 | Normal |
| Normals | Neb | 8 | 68 | 34 | 25 | 13 | 5 | Normal |
| | Dun | 14 | 75 | 57 | 25 | 9 | 3 | Normal |
| | Mau | 9 | 85 | 56 | 28 | 8 | 1 | Normal |
| | Mar | 10 | 81 | 65 | 28 | 11 | 3 | Normal |
| | Hug | 11 | 99 | 71 | 33 | 17 | 3 | Normal |
| | Cor† | 12 | 78 | 37 | 23 | 10 | 2 | Convalescent hepatitis |
| | Mean | 10.6 | 75.7 | 51.0 | 24.7 | 11.3 | | |
| | S.D.±‖ | 2.6 | 28.7 | 10.7 | 4.3 | 3.1 | | |
| | $S.D._m$± | .8 | 9.1 | 3.4 | 1.4 | 1.0 | | |

* Infusion lasted almost 60 minutes.
†Patients with hepatitis studied on two occasions.
‡ Fifty mg. infusion.
§ Icterus index >100.
‖ S.D. Standard Deviation.
$S.D._m$ Standard Deviation of Mean.

rate occasions as their hepatitis improved. It can be seen that the rate of removal increased as the liver disease became less severe (Figure 2).

It should be noted that, in patient War (Table II), who had metastatic carcinoma of the liver, the rate of steroid removal was only moderately reduced, although the icterus index was greater than 100.

Several patients with advanced cirrhosis of the liver, as evidenced by clinical findings and biopsy, showed impairment of the mechanism for removing the 17-hydroxycorticosteroids from the plasma even though the BSP retention at 45 minutes was only slightly elevated. In the other cases, however, there was good correlation between the BSP retention at 45 minutes and the impaired rate of 17-hydroxycorticosteroid removal. This is illustrated in Figure 3 where the plasma level of the 17-hydroxycorticosteroids at four hours is plotted against the BSP retention at 45 minutes. The four-hour level was chosen because there was a maximum spread of values at this time. The coefficient of correlation is $+0.89 \pm .04$ ($p < .01$).

### *The plasma 17-hydroxycorticosteroid level after ACTH stimulation*

The average plasma 17-hydroxycorticosteroid levels of eight patients with cirrhosis of the liver during a 6-hour intravenous infusion of 25 mg. of ACTH are compared in Table III with the

TABLE III

*Effect of ACTH on plasma 17-hydroxycorticosteroid levels*

| | γ 17-hydroxycorticosteroid/100 ml. plasma | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | 2 hrs. | 4 hrs. | 6 hrs. | 8 hrs. | 10 hrs. | 12 hrs. | 24 hrs. |
| Cirrhosis | 14.3 ± 2 | 32.4 ± 3 | 41.9 ± 4.4 | 45.0 ± 3.8 | 39.3 | 28.5 | 18.0 | 13.6 |
| Normal (8) | 10 ± .5 | 26 ± 1 | 36 ± 2 | 42 ± 2 | — | — | — | — |

TABLE IV

*The urinary excretion of 17-hydroxycorticoids*

| | No. of subjects | No. of days | Free mg./day | Conjugated mg./day | % Conjugated |
|---|---|---|---|---|---|
| Normals | 8 | 11 | .16 (.08–.30) | 5.3 (2.0–12.2) | 97.2 (91.5–98.5) |
| Liver disease | 9 | 15 | .15 (.03–.51) | 3.3 (.39–5.4) | 95.7 (79.6–99.3) |

levels in a group of normal subjects who received similar infusions.

It is apparent that there was no significant difference in the levels in the two groups during the 6-hour test. The ACTH infusions caused a mean rise in the plasma levels in the patients with cirrhosis of 30.7 μg, as compared with 32 μg in the normal group.

### *The plasma 17-hydroxycorticosteroid levels after the infusion of tetrahydrocortisone*

The 17-hydroxycorticosteroid levels of the plasma after the intravenous infusion of tetrahydrocortisone, 1 mg. per Kg., are plotted in Figure 4. It is evident that this material was cleared much more rapidly than hydrocortisone, both in the normals and in the patients with liver disease. In the four patients studied, two with cirrhosis of the liver and two normal subjects, the levels had returned to the normal range in about 90 minutes. In one subject with biliary cirrhosis, who was being treated by means of biliary drainage, the rate of 17-hydroxycorticosteroid removal was more rapid than in the two normal subjects.

### *The urinary excretion of free and conjugated 17-hydroxycorticoids*

The results of the daily urinary excretions of 17-hydroxycorticoids during the control period are summarized in Table IV. Although the urinary excretion of the free compound was the same in the two groups, the patients with liver disease excreted less 17-hydroxycorticoids in the conjugated form than did the normal subjects.

The increased excretion of total 17-hydroxycorticoids per 24 hours, occasioned by the infusion of hydrocortisone, in the normal subjects averaged 19.6 per cent (11.0 to 26.0 per cent) of the amount infused and 18.2 per cent (15.2 to 25.2 per cent) in the subjects with liver disease (as

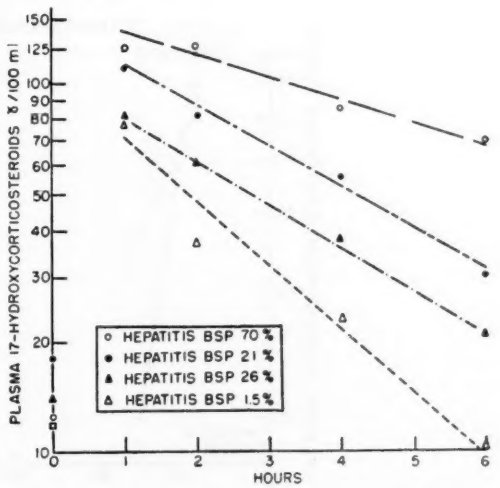


FIG. 2. THE PLASMA 17-HYDROXYCORTICOSTEROID LEVELS OF TWO PATIENTS WITH HEPATITIS AFTER THE STANDARD HYDROCORTISONE INFUSION

Two infusions were carried out on each patient as his hepatitis improved. The circles refer to patient Pur and the triangles to patient Cor (Table II).

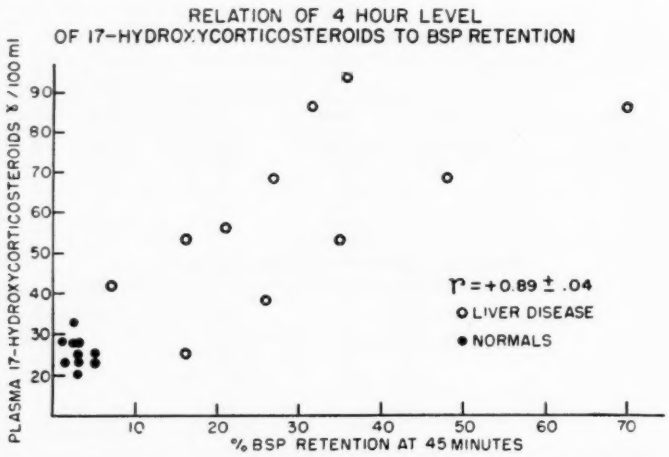
RELATION OF 4 HOUR LEVEL
OF 17-HYDROXYCORTICOSTEROIDS TO BSP RETENTION
PLASMA 17-HYDROXYCORTICOSTEROIDS γ / 100 ml
% BSP RETENTION AT 45 MINUTES
r = +0.89 ± .04
LIVER DISEASE
NORMALS

FIG. 3. THE RELATION OF BSP RETENTION AND THE FOUR-HOUR PLASMA 17-HYDROXYCORTICOSTEROID LEVEL AFTER THE STANDARD HYDROCORTISONE INFUSION

measured in milligrams of cortisone). These data are tabulated in Table V.

On the day of infusion, the patients with liver disease excreted larger amounts of the 17-hydroxycorticoids in the free form than the normal subjects. In two patients (one normal subject and one patient with hepatitis), urines were collected at 6-hour intervals on the day of the hydrocortisone infusion. In this study, the normal subject excreted practically all of the free steroid during the first 6-hour period whereas the subject with liver disease excreted a significant portion of

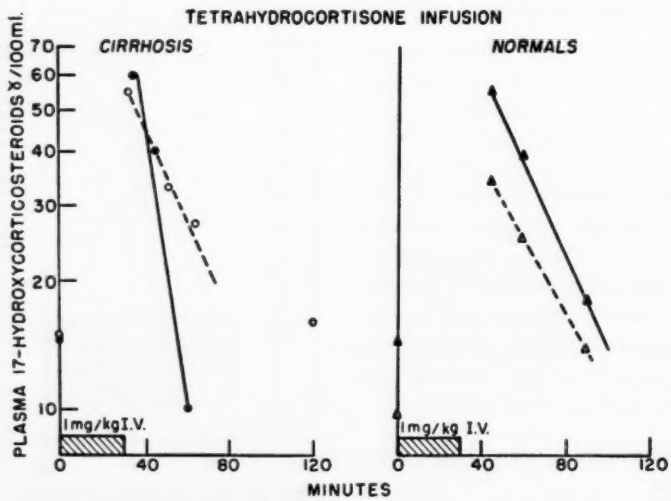


FIG. 4. THE PLASMA 17-HYDROXYCORTICOSTEROID LEVELS AFTER THE INTRAVENOUS INFUSION OF TETRAHYDROCORTISONE 1 MG. PER KG. OVER A 30-MINUTE PERIOD

The steeper curve on the left depicts a study in a female patient with biliary cirrhosis whose bile was being drained externally.

TABLE V

*Urinary excretion of 17-hydroxycorticoids after hydrocortisone infusion*

| | Patient | Dose infused mg. | Excretion: Control day mg./day total 17-hydroxycorticoid | Excretion: Day of infusion mg./day total 17-hydroxycorticoid | Excretion: Increase mg./day total 17-hydroxycorticoid | % Dose excreted |
|---|---|---|---|---|---|---|
| Liver disease | Mee | 50 | 4.4 | 13.8 | 9.4 | 18.8 |
| | Tur | 50 | .9 | 13.9 | 13.0 | 26.0 |
| | Pur | 66 | 3.4 | 15.2 | 11.8 | 17.9 |
| | Phi | 59 | 3.0 | 9.5 | 6.5 | 11.0 |
| | Jep | 91 | 4.8 | 16.7 | 11.9 | 13.1 |
| | War | 81 | 3.3 | 21.5 | 18.2 | 22.5 |
| | Pur | 68 | 4.7 | 16.5 | 11.8 | 17.3 |
| | Qui | 60 | 1.9 | 11.0 | 9.1 | 15.1 |
| | Cor | 59 | 4.8 | 17.9 | 13.1 | 22.2 |
| Mean | | | 3.5 ± .4 | | | 18.2 ± 1.1 |
| Normal | Neb | 50 | 12.3 | 24.9 | 12.6 | 25.2 |
| | Sha | 50 | 9.5 | 19.2 | 9.7 | 19.4 |
| | Car | 89 | 2.8 | 21.5 | 18.7 | 21.0 |
| | Mar | 87 | 5.5 | 21.5 | 16.0 | 18.4 |
| | Dun | 61 | 3.7 | 15.4 | 11.7 | 19.2 |
| | Mau | 64 | 6.0 | 19.7 | 13.7 | 21.4 |
| | Hug | 51 | 2.1 | 11.1 | 9.0 | 17.6 |
| | Cor | 60 | 4.8 | 13.9 | 9.1 | 15.2 |
| Mean | | | 5.8 ± 1.2 | | | 19.6 ± 1.2 |

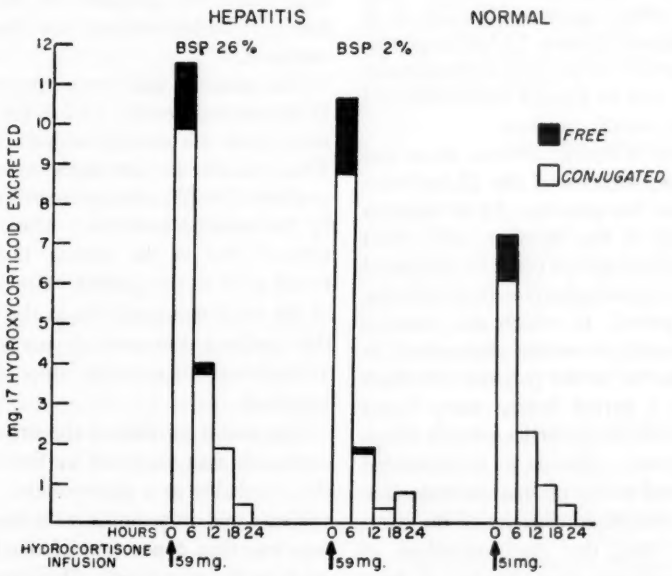


FIG. 5. THE URINARY 17-HYDROXYCORTICOID EXCRETION AFTER THE STANDARD HYDROCORTISONE INFUSION IN A PATIENT WITH HEPATITIS COMPARED WITH THAT IN A NORMAL SUBJECT

The patient with hepatitis was studied on two occasions to note the effect of improvement in liver function. As liver function improved, there was less free 17-hydroxycorticoid excreted during the second six-hour period.

free steroid during the second 6-hour period. This is illustrated in Figure 5.

In the four patients studied following the infusion of tetrahydrocortisone, 20 to 30 per cent of the infused steroid could be recovered, largely in the six hours following infusion.

## DISCUSSION

The foregoing data demonstrate that there is a high degree of correlation between the rate of 17-hydroxycorticosterone (hydrocortisone) disappearance from the plasma and the clearance of sulfobromphthalein (BSP). These results suggest that the liver plays an important role in the catabolism of hydrocortisone as well as of other steroid hormones (1). Since there is evidence that 17-hydroxycorticosterone is the principal hormone secreted by the human adrenal cortex (2), it would appear that the circulating level of the adrenocortical hormones is regulated not only by the degree of stimulation of the adrenal cortex but by the functional state of the liver. In view of the fact that liver function is depressed during surgery (9) and other stresses (10–12), it is likely that the elevated plasma 17-hydroxycorticosteroid level observed under these circumstances may be ascribed in part to adrenal stimulation and in part to impaired hepatic function.

After the infusion of hydrocortisone, there was an initial rapid disappearance of the 17-hydroxycorticosteroids from the plasma. At 60 minutes after the beginning of the infusion, only about 5 per cent of the infused steroid could be accounted for by the 17-hydroxycorticosteroids in the plasma. After the initial period, in which the material was cleared very rapidly from the plasma both in the normal subjects and in the patients with liver disease, there was a period lasting many hours during which the 17-hydroxycorticosteroids disappeared at a slower rate. This, as we have pointed out, was proportional to the plasma concentration and related to the functional capacity of the liver.

It would seem, then, that the metabolism of exogenous hydrocortisone must involve at least three stages. First, there is a very rapid phase which removes more than 90 per cent of the material from the plasma in a matter of minutes; then, there is a phase in which the corticosteroids disappear in a logarithmic fashion. This phase is influenced by the function of the liver. Lastly, there must be another reaction which results in the material being excreted as a conjugate of tetrahydrocortisone.

The fate of the infused 17-hydroxycorticosteroid demands further study, but there are several possibilities. There is evidence that a portion is excreted into the small bowel *via* the liver and biliary tract (13). It is possible that some goes into the connective tissues (14). In addition, it should be noted that that portion in which the characteristic side chain has been modified or conjugated is not measured by our techniques. Recently, it has been shown that, in a variety of circumstances, tetrahydrocortisone is the principal 17,21-hydroxycorticoid in the urine (15). It is presumed that this metabolically inert compound is a major urinary excretion product of the adrenocortical hormone. Since we found that tetrahydrocortisone was cleared from the blood stream in both normal subjects and in patients with severe liver disease within one to two hours, it is unlikely that the elevated 17-hydroxycorticosteroid level which was present two hours after the infusion of hydrocortisone was due to tetrahydrocortisone.

The patients with liver disease excreted less 17-hydroxycorticoids ($3.5 \pm 0.4$ mg.) per 24 hours than the normal subjects ($5.5 \pm 1.2$ mg.). These results do not agree with those of other workers (16–20) who measured urinary corticoids by less specific methods. Although the ACTH-induced rise in the plasma 17-hydroxycorticosteroid level in the patients with liver disease was of the same magnitude as in the normal subjects, this implies a decreased adrenocortical secretion of 17-hydroxycorticosterone since its removal was impaired.

The major portion of the urinary 17-hydroxycorticoids was excreted in the conjugated fraction, probably as a glucuronide. In both normal subjects and in patients with liver disease, there was less than 5 per cent of the 17-hydroxycorticoids in the free form. However, on the day of the hydrocortisone infusion, there was an increase in the free steroid fraction excreted. This was more marked in the patients with liver disease than in the normal subjects. This increased amount probably was the result of the higher plasma levels

rather than because of saturation of the subjects' conjugating capacity.

It is well to emphasize that there are certain problems in relation to the determination of the absolute amounts of 17-hydroxycorticosteroids in the blood and urine. For the blood determinations, hydrocortisone was used as the standard of reference both for the hydrocortisone and tetrahydrocortisone infusions, and cortisone was used as the reference standard for the urine determinations since we have no information in regard to the relative proportions of cortisone, hydrocortisone and tetrahydrocortisone present under the conditions of the study. The intensity of color produced in the Porter-Silber reaction decreases with the above compounds in the order given (21).

This study again emphasizes the fine adjustment of the long-term homeostatic mechanisms (10) controlling the 17-hydroxycorticosteroid level of the plasma. Despite the markedly slower catabolism of hydrocortisone in the subjects with liver disease, the 8 A.M. levels were approximately the same in the normal subjects as in those with liver disease. Since the hormone is catabolized more slowly in the patients with liver disease, less secretion is needed to maintain the plasma level. This conclusion is supported by the observation that less hydroxycorticoid is excreted in the urine.

Finally, it should be pointed out that the metabolism of the 17-hydroxycorticosteroids at more physiological levels may not be quite the same as at the high levels induced in the present study.

## SUMMARY

1. Twelve patients with liver disease and eleven normal subjects were given an infusion of 17-hydroxycorticosterone (hydrocortisone) intravenously and the plasma levels and urine excretions of the 17-hydroxycorticosteroids were measured. A similar study was made with tetrahydrocortisone.

2. The disappearance of the 17-hydroxycorticosteroids from the plasma was a first order reaction for both substances. The rate of disappearance of hydrocortisone was inversely proportional to the degree of liver damage as measured by BSP retention while tetrahydrocortisone disappeared at a much more rapid rate which was independent of liver function.

3. The urinary 17-hydroxycorticoid excretion on the day of hydrocortisone administration accounted for only about 20 per cent of the material administered in both the normal subjects and in the patients with liver disease. About 90 to 95 per cent of the urinary 17-hydroxycorticoids excreted were conjugated.

4. The rise in the plasma levels of 17-hydroxycorticosteroids in response to ACTH stimulation in patients with liver disease was normal.

5. The patients with liver disease had normal fasting levels of 17-hydroxycorticosteroids in the plasma but excreted less in the urine than did normal individuals.

6. The elevated 17-hydroxycorticosteroid level in stress must be considered as resulting both from increased adrenocortical stimulation and impaired removal by the liver.

7. Since patients with liver disease excrete less 17-hydroxycorticoids in the urine than do normal subjects despite the normal 8 A.M. plasma levels, there would seem to be an homeostatic mechanism which results in decreased adrenocortical secretion in the subjects with liver disease in whom the rate of 17-hydroxycorticosteroid removal from the plasma is impaired.

## ACKNOWLEDGMENTS

The authors wish to acknowledge the faithful services of the metabolic ward group, Mrs. J. Nicoll, Mr. A. Curtis, and Mrs. E. Cook, the technical assistance of Mr. E. Simons and Mr. P. Sine of the research laboratory, the material assistance of the Merck Company for supplies of hydrocortisone and of the Armour Company for supplies of ACTH.

We also wish to thank Dr. M. M. Wintrobe for his helpful advice and criticism of the manuscript.

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# QUANTITATIVE ASPECTS OF IODINE METABOLISM. THE EXCHANGEABLE ORGANIC IODINE POOL, AND THE RATES OF THYROIDAL SECRETION, PERIPHERAL DEGRADATION AND FECAL EXCRETION OF ENDOGENOUSLY SYNTHESIZED ORGANICALLY BOUND IODINE

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(Submitted for publication June 2, 1954; accepted July 7, 1954)

Of the three important quantitative aspects of iodine metabolism-thyroidal iodide accumulation, release of organically bound iodine from the thyroid and metabolism of organic iodine in the body tissues, only the first has been measured adequately in man (1). The purpose of the present report is to outline methods for the quantitative evaluation of: 1) The organic iodine pools of the body; 2) the rate of thyroidal secretion of organically bound iodine; 3) the metabolic degradation and fecal excretion of organically bound iodine;[1] and 4) exogenous and endogenous iodine balance.

The technical methods employed and the schematic model on which the analyses are based are common to all the determinations and are presented first. The specific analyses and results obtained for the various phases of iodine metabolism are then considered in separate sections. An attempt has been made to follow a similar order of presentation in each of these sections. The theoretical basis for the methods employed in the measurement of each aspect of iodine metabolism is followed by a sample calculation from the observed data in one of the subjects, R. D. Reference is then usually made to the results of the measurements, and the section is concluded with an analysis of the validity of the determinations and the sources of error.

## TECHNICAL METHODS

Subjects were hospitalized euthyroid and hyperthyroid patients. In all cases the diagnoses were confirmed by several observers and considered established without reference to the results obtained in the radioactive iodine tests. One hundred to two hundred $\mu$c of $I^{131}$ were administered intravenously, as carrier-free sodium iodide. At intervals thereafter, radioactivity in the thyroid, blood and urinary and fecal excretions was assayed. A bismuth-walled Geiger tube was used for the thyroid assays according to methods previously described (2) and a well-type scintillation counter was employed for assays of radioactivity in blood and excreta. Five ml. samples of plasma or packed erythrocytes were counted with a sensitivity of $1.03 \times 10^6$ counts per minute per $\mu$c $I^{131}$ above a background of about 200 counts per minute. Two hundred ml. samples of urine were counted in a constant geometrical relationship to the scintillation crystal with a sensitivity of $8.3 \times 10^4$ counts per minute per $\mu$c $I^{131}$ above a background of about 300 counts per minute. Twenty-four hour stool collections were made up to 300 ml. volumes with water, homogenized in an Oster mixer, and counted in a similar manner with a sensitivity of $3.7 \times 10^4$ counts per minute per $\mu$c $I^{131}$. $PBI^{127}$ in plasma was determined by the method of Barker, Humphrey, and Soley (3).[2]

$PBI^{131}$ and $iodide^{131}$ in plasma were determined from assays of plasma and packed red cells utilizing the plasma: erythrocyte ratio of $iodide^{131}$ (usually 1.6 to 1.8) for each subject and the observation that $PBI^{131}$ is not detectable in packed cells when allowance is made for trapped plasma. In many instances 10 per cent cold trichloracetic acid was added to the plasma and precipitate and filtrate were separately assayed. However, since it was observed that trichloracetic acid frequently precipitated significant quantities of $I^{131}$ as iodide added to plasma, the method described above was considered more reliable for the quantitative separation of iodide and PBI. Complete urine and stool collections were made during the periods of observation in most of the cases. After there appeared to be equilibration of the $I^{131}$ in the exchangeable organic iodine pool, 1-methyl-2 mercaptoimidazole (tapazole) was administered in doses of 25 to 30 mgm. every six hours, which are sufficient to inhibit thyroidal iodide binding almost completely (4).

In another type of experiment, the concentration of $PBI^{131}$ in the plasma was followed for about 10 days in three subjects who received labeled plasma from a donor treated with 8.5 mc. $I^{131}$ for Graves' disease.

[1] This process has occasionally been referred to as hormonal iodine utilization. Since it is questionable whether the organic iodine released by the thyroid represents homogeneous hormonally active substance or whether all organic iodine which undergoes degradation is utilized in any strict metabolic sense, it seems preferable to use the more general term, organic iodine degradation, in place of hormonal iodine utilization.

[2] We are indebted to Dr. Solomon Silver and to Mr. Herbert Lew in the laboratory of Dr. Bruno Volk for these determinations.

SCHEMATIC MODEL

For the purpose of orientation, the major pathways of iodine transport are summarized briefly with reference to the schematic model shown in Figure 1. Intravenously administered radioiodide is distributed rapidly among the iodide pools of blood, tissue fluids, and thyroid gland. Clearance of the total iodide pool is effected by binding in the thyroid and excretion in the urine. Organically bound $I^{131}$ released from the thyroid into the plasma and tissue fluids is subject to metabolic degradation, the principal end product being iodide which then repeats the cycle. A small amount of non-protein bound organic iodine is also excreted by the kidneys (5) but usually comprises only a small fraction of the total urinary iodine. There is also a loss of organic iodine in the stool which may represent only a fraction of the total iodine transfer through the enterohepatic circulation (6). Since virtually the entire administered dose of $I^{131}$ was accounted for in the urinary and stool excretions in our cases followed for prolonged periods, no other major excretory pathway need be considered. The radioiodine retained in the body at any time ($I^{131}_{ret}$) is then determined by subtracting the cumulative urinary and fecal excretions from the dose administered.

In normal and hyperthyroid subjects, the total iodine present in the enterohepatic circulation and in the iodide fraction of the thyroid and extrathyroidal tissues at any time represents only a small part of the total iodine content of the body. Therefore, following initial clearance of the administered radioiodide, the major portion of the radioiodine in the body may be considered to be undergoing distribution between two compartments, the thyroidal and extrathyroidal organic iodine pools (Figure 2), which are in

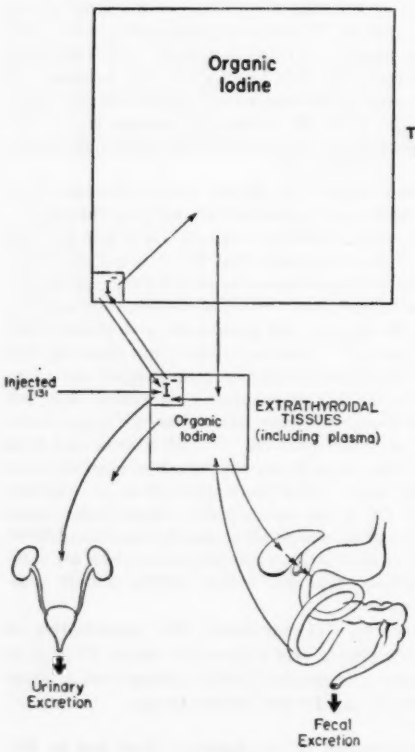


FIGURE 1

See text for discussion.

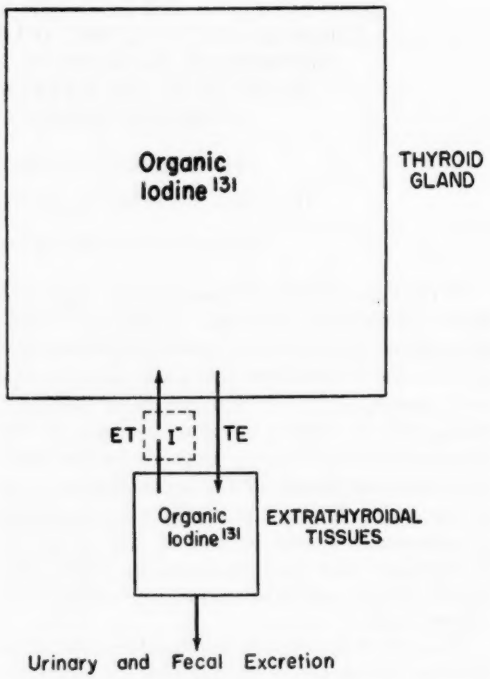


FIGURE 2

Following peak thyroidal uptake and renal excretion of the administered iodide$^{131}$, the radioactive iodine remaining in the body is almost entirely in an organic form undergoing distribution between thyroidal and extrathyroidal organic iodine pools. See text for discussion.

dynamic equilibrium. A transient iodide phase is interposed in the pathway from extrathyroidal to thyroidal compartments.

## PART I—ESTIMATION OF THE ORGANIC IODINE POOLS OF THE BODY

These determinations are based on the assumption that when a tracer substance has mixed uniformly in all its compartments of distribution, the specific activity is the same in all these compartments and, in the absence of turnover, remains constant. In the present section radioactivity is treated only in terms of the $I^{131}$ remaining in the body ($I^{131}_{ret}$) thus obviating the necessity for consideration of turnover of the pool with newly ingested iodine.

### A. *The total exchangeable organic iodine pool*

Following peak uptake by the thyroid, the curves of thyroidal radioactivity and plasma concentration of $PBI^{131}$, plotted as fractions of $I^{131}_{ret}$ *vs.* time, each approach an horizontal asymptote (Figures 3a, 4). The maintenance of horizontal plateaus in these curves over a period of several weeks indicates that the radioiodine tracer has mixed uniformly in an organic iodine pool which remains constant at least over this period of time, and which, therefore, has been called "the exchangeable organic iodine pool." This pool is calculated from the ratio $\frac{PBI^{127}/\text{liter plasma}}{\text{fraction } I^{131}_{ret}/\text{liter plasma}}$ at distribution equilibrium.

*Calculation*—In R. D. (Figure 3a), distribution equilibrium was attained by the 7th day, at which time the fraction $I^{131}_{ret}$ per liter of plasma was .015. Since $PBI^{127}$ was 154 micrograms per liter, the total exchangeable organic iodine pool $= \frac{154 \text{ micrograms/liter}}{.015/\text{liter}}$ $= 10{,}300$ micrograms.

In most untreated hyperthyroid and euthyroid subjects the total exchangeable organic iodine pool ranged from about 7000 micrograms to 13,000 micrograms with a few almost as high

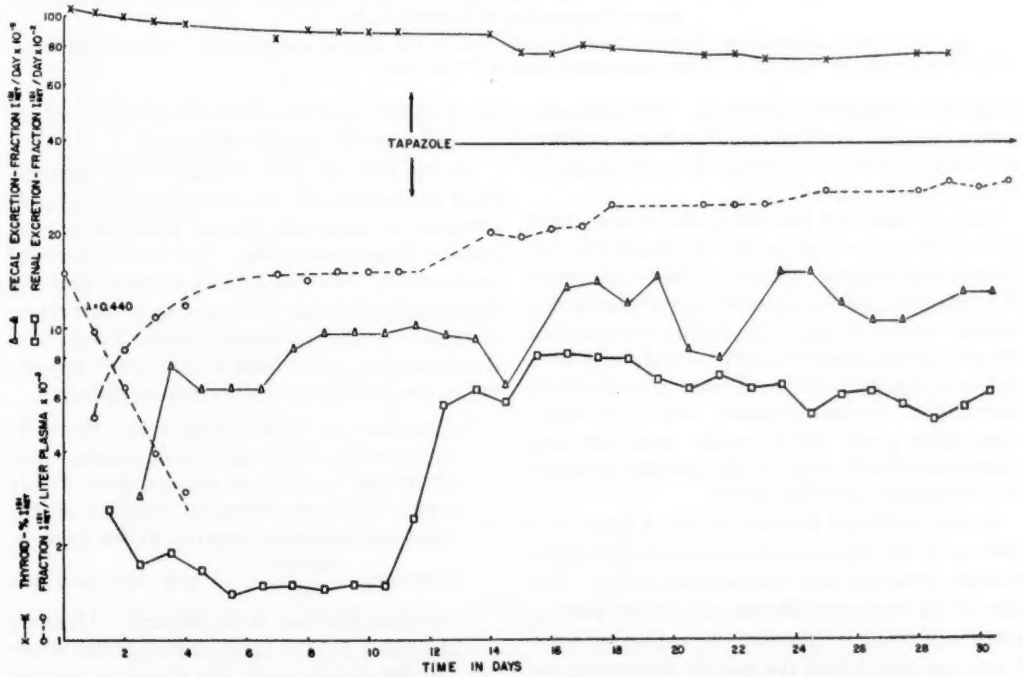


FIG. 3A. RADIOACTIVITY (FRACTION $I^{131}_{ret}$) IN THYROID AND PLASMA AND IN URINARY AND FECAL EXCRETIONS IN SUBJECT R. D.

The urine excretions are plotted at mid-day.

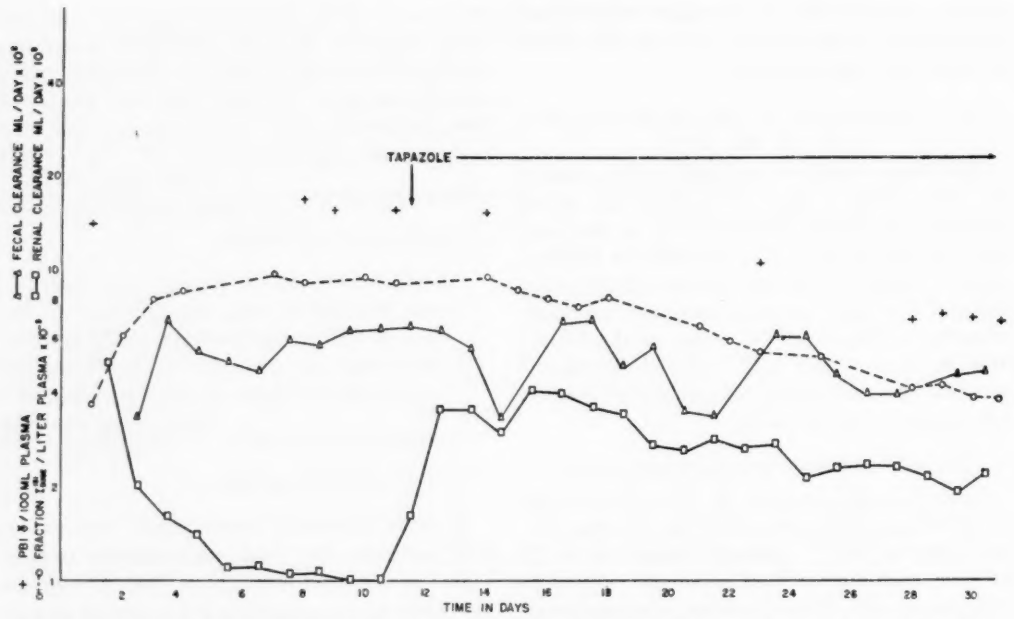


FIG. 3B. PLASMA CONCENTRATIONS OF $PBI^{131}$ AND $PBI^{127}$ AND APPARENT FECAL AND RENAL PLASMA ORGANIC IODINE CLEARANCES IN PATIENT R. D.

The latter were determined by dividing the daily excretions by the plasma concentrations. Plasma $PBI^{131}$ concentrations are plotted as fractions of the administered dose of $I^{131}$ per liter.

as 20,000 micrograms (Table I). The pool sizes were generally much reduced in those subjects who had been treated previously with tapazole, radioiodine or surgery.

Inactive pools not entered by the administered radioiodine cannot, of course, be detected under these circumstances. However, the maintenance of a constant specific activity in the plasma for several weeks (Figure 5), during anti-thyroid therapy with tapazole, when thyroidal and plasma radioactivity may fall to 50 per cent or less of the control values, indicates that such unexchangeable pools which might exist are not readily mobilized, even in the presence of moderate depletion of iodine stores.

In one euthyroid subject (G. M., Figure 4), a plateau in the plasma concentration ($I^{131}_{ret}$) curve was not obtained even after several weeks. The size of his exchangeable organic iodine pool as given in Table I is therefore overestimated since it was calculated from the specific activity on the last day of observations, instead of the higher specific activity which would have been obtained at equilibrium.

B. *Thyroidal and extrathyroidal fractions of exchangeable organic iodine pool*

At the time of peak uptake of the administered radioiodide by the thyroid only negligible amounts of organically bound iodine have been released from the thyroid. The fall in thyroidal radioactivity (fraction $I^{131}_{ret}$) from peak uptake to the asymptotic value then represents the portion of organic iodine in extrathyroidal tissues and the asymptotic value itself is the portion remaining in the thyroid at distribution equilibrium.

*Calculation*—In R. D. (Figure 3a) thyroidal radioactivity fell from a peak uptake value of 108 per cent to an asymptote of 89 per cent. The extrathyroidal fraction of the total exchangeable organic iodine pool is, therefore, $\frac{108-89}{108} = 18$ per cent and the thyroidal fraction = 82 per cent. Utilizing the value for the total pool as given above (10,300 micrograms), the thyroidal and extrathyroidal pools are calculated to contain 8,450 micrograms and 1,850 micrograms respectively.

In euthyroid subjects not previously treated for hyperthyroidism (G. M. and R. F., Table I) the thyroid contained about 95 per cent and the extrathyroidal tissues about 5 per cent of the total exchangeable organic iodine pool. In untreated hyperthyroid subjects the extrathyroidal fraction generally ranged from 10 to 20 per cent of the total but in hyperthyroid and euthyroid subjects with reduced total pools (usually a result of previous therapy), the extrathyroidal compartment frequently contained 30 per cent or more of the total organic iodine (Table I).

The quantity of organic iodine in the extrathyroidal tissues was, of course, greater in hyper-

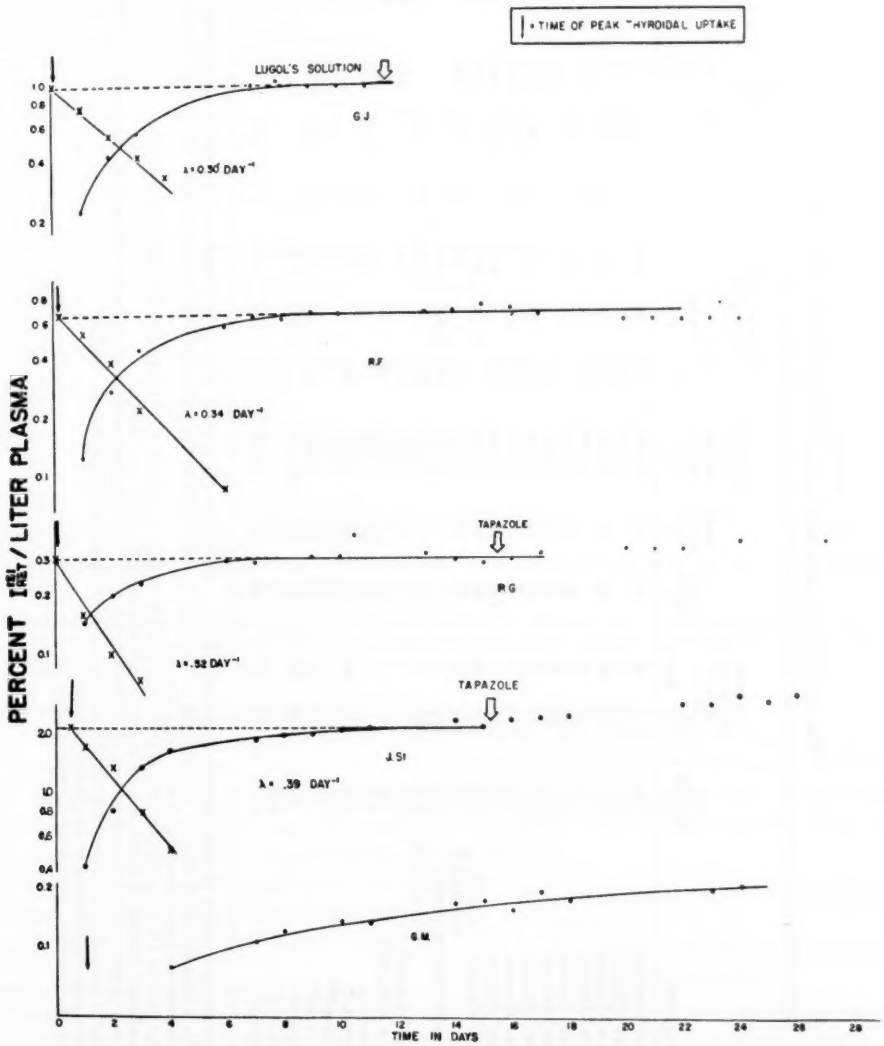


FIG. 4. PLASMA CONCENTRATIONS OF PBI$^{131}$ IN TERMS OF $I^{131}_{ret}$ AS A FUNCTION OF TIME

The rate of equilibration is found from the slope of the line joining the points of differences between the curve and the asymptotic value. $\lambda$ as given in the figure is equal to $\lambda_{ET} + \lambda_{TE}$ of the text. In G. M., equilibrium was not reached at the end of 24 days. In the remaining cases about one week was required for equilibration. See also R. D. in Figure 3a.

TABLE I

*Data pertaining to studies with endogenously synthesized organic iodine*

| Subject | Diagnosis | PBI μg./100 ml. | Iodide$^{131}$ clearances ml./min. Thyroid | Iodide$^{131}$ clearances ml./min. Renal | Peak* uptake % | Renal* excretion % | Exchangeable organic iodine pool μg. | Organic iodine degradation μg./day Method A | Method B | Method C | Fecal excretion μg./day | Thyroidal secretion μg./day From distribution kinetics | Thyroidal secretion μg./day After tapazole block | Fraction of pool Thyroidal | Fraction of pool Extrathyroidal | Extrathyroidal organic iodine space Liters | Extrathyroidal organic iodine space % Body wt. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R. D. | Graves' disease | 15.4 | 118 | 34 | 80 | 26 | 10,300 | 555 | 685 | 630 | 93 | 670 | 730 | .82 | .18 | 12.0 | 21 |
| J. St. | Graves' disease | 18.4 | 228 | 37 | | | 8,800 | 644 | 630 | | 81 | 450 | 664 | .85 | .15 | 7.2 | 11 |
| N. R. | Graves' disease | 14.4 | 185 | 30 | 80 | 18 | 10,600 | 670 | 750 | 570 | 65 | 860 | 820 | .86 | .14 | 10.1 | 14 |
| S. K. | Graves' disease | 7.4 | 120 | 48 | | | 19,500 | 296 | 260 | | 26 | | 420 | >.95 | <.05 | | |
| S. S. | Graves' disease | 18.7 | 460 | 31 | 93 | 10 | 4,150 | | 680 | 435 | 62 | 925 | | .69 | .31 | 7.0 | 11 |
| A. A. | Graves' disease | 11.4 | 56 | 30 | 65 | 24 | 12,600 | 485 | | | | | 570 | .90 | .10 | 10.7 | 15 |
| P. G. | Graves' disease | 13.1 | 345 | 25 | 97 | 11 | 9,380 | 445 | 970 | 670 | 41 | 600 | 495 | .92 | .08 | 6.0 | 12 |
| E. G. | Graves' disease | 14.8 | 236 | 27 | | | 17,600 | 590 | 860 | | 59 | 735 | 525 | .91 | .09 | 11.0 | 17 |
| W. R. | Graves' disease | 8.9 | 220 | | 108 | 10 | 7,100 | 360 | | 835 | | 460 | 465 | .85 | .15 | 12.1 | 22 |
| R. G. | Graves' disease—1st study | 19.2 | 181 | 29 | 95 | 14 | 11,600 | | 1,180 | 1,270 | | | 680 | .84 | .16 | 9.7 | 17 |
| | 2nd study† | 8.0 | 162 | 31 | 83 | 18 | 2,600 | 123 | 111 | 102 | 16 | 235 | 100 | .77 | .23 | 7.6 | 12 |
| G. J. | Graves' disease—1st study | 13.0 | 482 | 32 | 116 | 7 | 19,700 | 650 | 2,400 | 2,600 | | | | | | | |
| | 2nd study†‡ | 7.8 | 362 | 29 | 103 | 7 | 8,250 | 200 | 350 | 390 | 29 | 183 | | .92 | .08 | 8.5 | 12 |
| J. S. | Graves' disease† | 10.0 | | | 75 | 10 | 6,650 | 263 | | 260 | | | 335 | .84 | .16 | 10.6 | 20 |
| M. C. | Graves' disease†§ | 5.8 | | | 90 | 3 | 1,930 | 87 | | | | | 109 | .62 | .38 | | |
| T. L. | Euthyroid‖ | 6.8 | | | 21 | 65 | 2,400 | 87 | | 106 | 13 | 116 | 125 | .70 | .30 | 10.7 | 16 |
| C. B. | Euthyroid¶ | 4.4 | 31 | 41 | 44 | 50 | 6,500 | 108 | 77 | 83 | 9 | | | | | | |
| W. T. | Euthyroid** | 5.4 | | | 22 | 86 | 1,200 | 76 | | 68 | 5 | 83 | 77 | .64 | .36 | 8.0 | 18 |
| S. G. | Euthyroid** | 3.9 | 7 | 29 | 25 | 74 | 2,820 | 67 | 60 | 63 | 20 | 161 | 110 | .86 | .14 | 8.6 | 14 |
| G. M. | Euthyroid | 3.9 | 23 | 40 | 41 | 54 | <19,250 | | 107 | 119 | | | | >.96 | <.04 | | |
| F. B. | Euthyroid | 4.8 | | | 21 | 67 | | | | 40 | | | | | | | |
| R. F. | Euthyroid | 6.0 | 37 | 21 | 65 | 35 | 9,200 | | 104 | 108 | | 175 | | .94 | .06 | 9.3 | 12 |

* Renal excretion is taken at time of peak thyroidal uptake. Values for the sum of peak uptake and renal excretion significantly greater than 100 per cent are attributable to experimental error. The thyroid assays were standardized using a paraffin-filled phantom neck for normal thyroid anatomy (2) which does not faithfully reproduce the geometries of very large glands.

† After eight weeks (R. G.), seven weeks (G. J.) and three weeks (J. S. and M. C.) of intermittent tapazole therapy.

‡ Lugol's solution instead of tapazole administered as blocking agent after equilibrium.

§ By the end of this study the patient was clinically hypothyroid. He relapsed within a few weeks following discontinuation of tapazole.

‖ Two months post-thyroidectomy for Graves' disease; has remained euthyroid for six months.

¶ Five years post-thyroidectomy for Graves' disease.

** Nine months post $I^{131}$ therapy for Graves' disease.

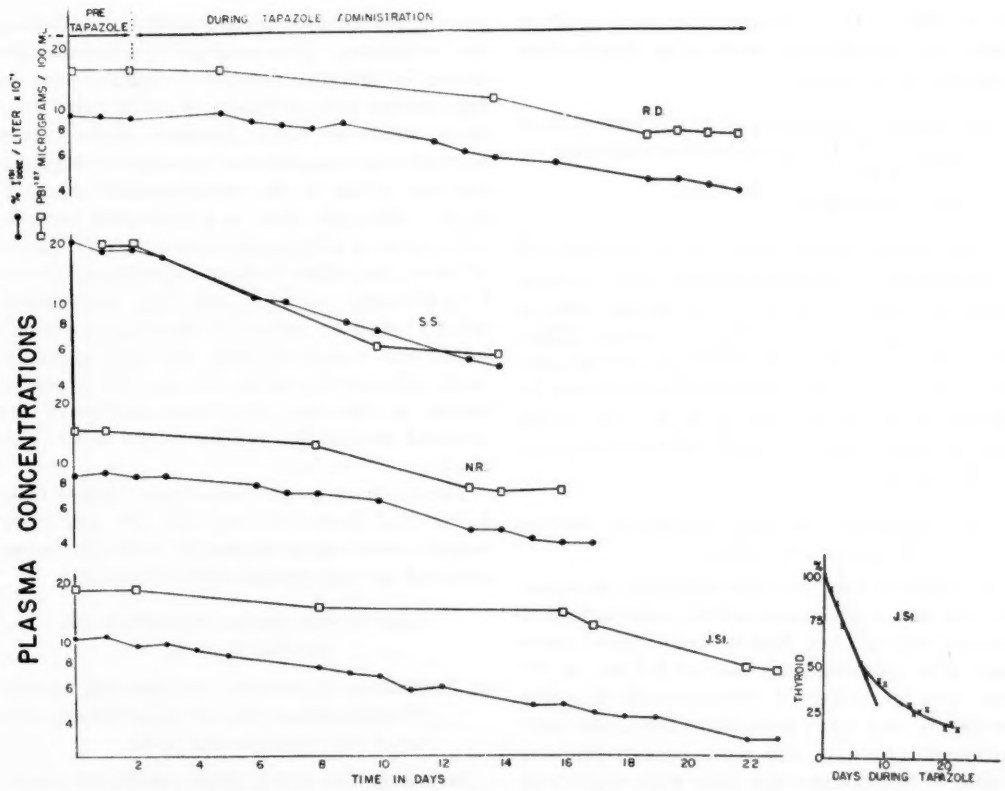


FIG. 5. PLASMA CONCENTRATIONS OF $PBI^{127}$ AND $PBI^{131}$ (PLOTTED AS PER CENT OF THE ADMINISTERED DOSE PER LITER) FOLLOWING ADMINISTRATION OF TAPAZOLE

Also shown is the thyroidal radioactivity of J. St. relative to the first day of tapazole administration. The ratio between $PBI^{127}$ and $PBI^{131}$ is not significantly different after 22 days of tapazole inhibition and the loss of over 80 per cent of thyroidal $I^{131}$.

thyroid than in euthyroid subjects and averaged about 570 micrograms (range, 400 to 750 micrograms) in the latter group.

The thyroidal pool alone may be evaluated after distribution equilibrium has been attained without knowledge of the $I^{131}$ retained, since thyroidal and plasma specific activities are then essentially the same[3] and thyroidal and plasma radioactivity and plasma $PBI^{127}$ are measurable. This determination suffers from uncertainty as to the time of distribution equilibrium, in the absence of data on $I^{131}$ retained. However, equilibrium of distribution may be presumed when thyroidal and plasma curves show a parallel decline.

C. *The extrathyroidal space of distribution of organic iodine*

1. *Estimations utilizing endogenously synthesized organic iodine*

Of the $PBI^{131}$ which is retained in the body, that which is not in the thyroid is distributed in the extrathyroidal tissues. Therefore, the apparent volume of distribution may be calculated from the expression $\frac{\text{fraction } I^{131}_{Tot} \text{ in extrathyroidal pool}}{\text{fraction } I^{131}_{Tot} \text{ per liter plasma}}$

[3] Theoretically the specific activities are never identical except at a single point (7), since excretion occurs solely from the extrathyroidal pool. However, since the rate of excretion of the total exchangeable pool is only about 1½ per cent per day as compared to half times of 1 to 3 days for equilibrium between thyroidal and extrathyroidal pools, the specific activities are virtually identical after the plasma peak is reached.

at any time. The experimental error is smallest when the estimation is made after distribution equilibrium is attained.

*Calculation*—Substituting the values calculated above in R. D., the extrathyroidal space = $\frac{0.18}{.015/\text{liter plasma}} = 12$ liters.

The extrathyroidal space of distribution of endogenously synthesized organic iodine ranged between 6 and 12 liters (11 to 22 per cent of body weight) with no evident significant differences between euthyroid and hyperthyroid subjects (Table I). The individual figures may be subject to errors as great as 30 per cent, being derived from relatively small differences in thyroidal radioactivity.

2. *Estimations utilizing exogenously biosynthesized organic iodine*

In order to evaluate more precisely the space of distribution of organic iodine, plasma from a patient with Graves' disease was obtained three days after a therapeutic dose of 8.5 mc. of $I^{131}$ and was administered intravenously to three recipients, one with hyperthyroidism, one with euthyroidism, and one with hypothyroidism. Table II summarizes the data with respect to this experiment and Figure 6 shows the plasma concentration curves for about 10 days following the injections. The terminal exponential decreases in concentration are presumably due to degradation and excretion of undegraded hormone, while the earlier portions of the curves represent the simultaneous processes of degradation and mixing in the extrathyroidal compartment. Although there is a continued increase in the space of distribution over a period of about 48 hours, the major fraction is distributed within 4 to 6 hours. In each case, the approximate volume of distribution of exogenous organic iodine was calculated from the total administered radioactivity divided by the $PBI^{131}$ concentration at the zero time extrapolation of the terminal straight line portion of the curve (Figure 6).

The volumes in the three cases ranged from 8.7 to 11.7 liters (10.3 to 20.6 per cent body weight) which agree reasonably with the values obtained for endogenous organic iodine.

## PART II—THYROIDAL SECRETION OF ORGANIC IODINE

### A. *Estimation of secretion rate from the kinetics of distribution of organic iodine between thyroidal and extrathyroidal pools*

Assuming that steady state conditions obtain during the course of the experiment, analysis of

TABLE II

*Data pertaining to distribution and degradation of exogenously synthesized organic iodine* *†

| Subject | Diagnosis | Counts/min. transfused | Date admin. | PBI μg./100 ml. | Space of distribution | | Extrathyroidal pool—organic iodine μg. | Turnover rate %/day | Rate of degradation‡ μg./day |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Liters | % Body wt. | | | |
| R. A. | Hypothyroid (post thyroidectomy for carcinoma) | $3.42 \times 10^6$ | 5/25/53 | 1.7 | 8.7 | 10.3 | 148 | 9.76 | 13 |
| F. S. | Euthyroid | $2.78 \times 10^6$ | 5/25/53 | 6.7 | 9.1 | 12.1 | 610 | 12.4 | 68 |
| G. Mi. | Hyperthyroid (intermittent therapy with tapazole for several weeks prior to study) | $5.93 \times 10^6$ | 5/26/53 | 11.2 | 11.7 | 20.6 | 1,310 | 22.0 | 256 |

* Donor, M. C. (Graves' disease) was given 8.45 mc. $I^{131}$ on 5/22/53. On 5/25, 98.5 per cent of radioactivity in plasma was precipitated by 10 per cent cold trichloracetic acid; 72.6 per cent was butanol soluble; 26.6 per cent was present in the butanol insoluble fraction. $Na_2CO_3$ and NaOH extract of the butanol soluble fraction contained 2 per cent of the total radioactivity.

† Recipients were maintained on 25 mg. tapazole every six hours to prevent reaccumulation of degraded iodine by the thyroid. 92 to 102 per cent of the radioactivity in the plasma samples of the recipients was precipitable with 10 per cent cold trichloracetic acid.

‡ Turnover was assumed to be due to degradation plus fecal excretion. The rates of degradation were calculated by estimating fecal excretion of undegraded hormone at 12½ per cent of the rate of degradation.

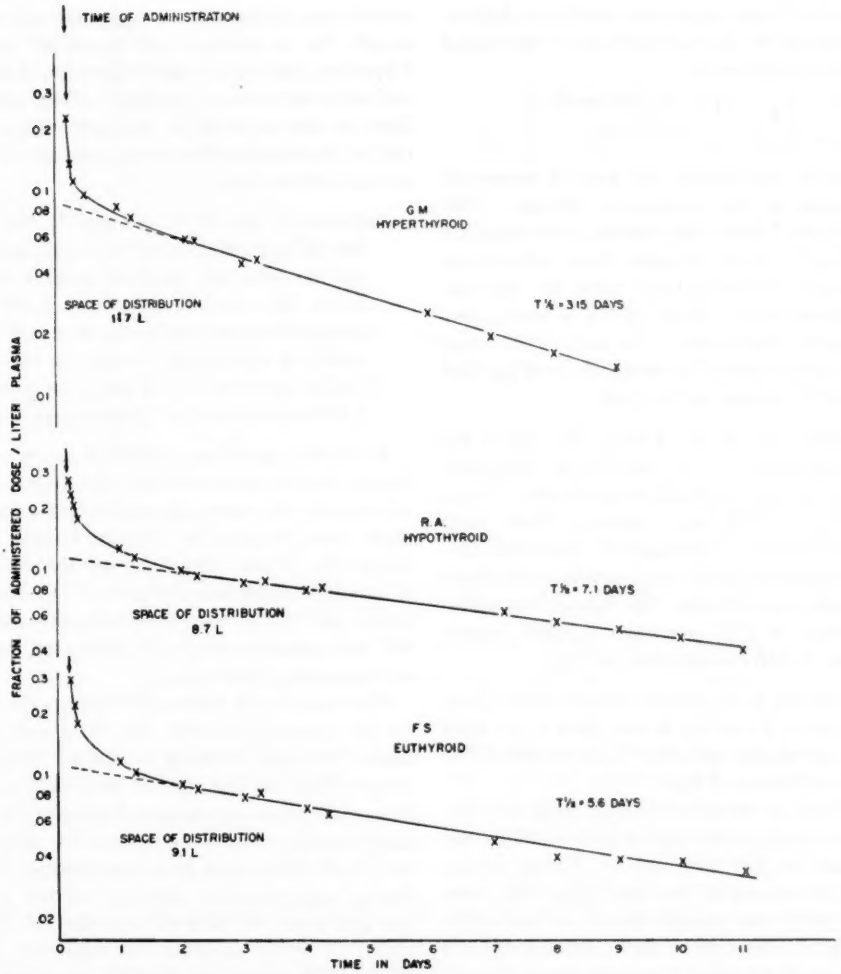


FIG. 6. PLASMA CONCENTRATION CURVES OF RADIOACTIVITY AS A FUNCTION OF TIME FOLLOWING THE INTRAVENOUS INJECTION OF EXOGENOUSLY SYNTHESIZED ORGANIC IODINE (SEE TABLE II)

The 15 minute spaces of distribution approximate the plasma volumes.

the kinetics of distribution of $PBI^{131}$ between thyroidal and extrathyroidal compartments permits a reasonable estimate of the rate of organic iodine secretion from the thyroid to be made in many cases (Table I).

Let T and E be the radioactivity in terms of fraction $I^{131}_{ret}$ in the thyroidal and extrathyroidal organic iodine pools respectively at any time, t, following peak uptake. Let $\lambda_{TE}$ and $\lambda_{ET}$ be the rate constants of transfer between thyroidal and extrathyroidal pools in the order of the subscript designations. Then,

$$\frac{dT}{dt} = -\lambda_{TE}T + \lambda_{ET}E$$

$$\frac{dE}{dt} = -\lambda_{ET}E + \lambda_{TE}T$$

On substituting,

at $t = 0$ $\quad T = T_0 = 1, \quad E = 0$

and

at $t = \infty$ $\quad T = T_\infty, \qquad E = E_\infty = T_0 - T_\infty$

the solution of these equations yields the following expressions for the radioactivity in thyroidal and extrathyroidal pools.

$$T = T_\infty + (1 - T_\infty)e^{-(\lambda_{TE}+\lambda_{ET})t}$$
$$E = E_\infty(1 - e^{-(\lambda_{TE}+\lambda_{ET})t})$$

where $(\lambda_{TE} + \lambda_{ET})$ equals the rate of approach of the curves to the asymptotic values. This rate is obtained from the plasma concentration curves (Figures 3a and 4) since these values have much greater reliability than those for the thyroidal radioactivity. Since $\lambda_{ET}E_\infty = \lambda_{TE}T_\infty$, $\lambda_{TE}$ can be readily evaluated. The rate of thyroidal secretion is then given by the product of $\lambda_{TE}$ and the thyroidal organic iodine pool.

*Calculation*—In R. D. (Figure 3a), $\lambda_{ET} + \lambda_{TE} = 0.440$ days$^{-1}$. $E_\infty$ and $T_\infty$ as calculated above are 0.18 and 0.82, respectively. Then, $0.18\ \lambda_{ET} = 0.82\ \lambda_{TE}$. Solving from both equations $\lambda_{ET} = .079$ days$^{-1}$. Since the thyroidal organic iodine pool as calculated above is 8,450 micrograms, the rate of thyroidal secretion = .079 per day × 8,450 micrograms = 670 micrograms per day.

When the fall in thyroidal radioactivity (fraction $I^{131}_{ret}$) from $T_0$ to $T_\infty$ is less than 5 per cent or so, the calculated value for $E_\infty$ is too unreliable to justify estimates of $\lambda_{TE}$.

If distribution of organic iodine between thyroid and extrathyroidal tissues were significantly complicated by the time lag in mixing among the multiple spaces of the extrathyroidal compartment or by the possible failure of the radioiodine to mix uniformly in the functional thyroid pool, the analysis would require more than a single exponential component to describe the approach of the curves to the asymptotic values (8). However, in all the cases of this study, distribution appeared to take place at a single rate (Figures 3a, 4) and it may be recalled that mixing within the extrathyroidal pool (Figure 6) is quite rapid compared to distribution between thyroidal and extrathyroidal pools (Figure 4).

B. *Estimation of secretion rate from the decrease in thyroidal radioactivity during tapazole inhibition*

If the thyroid is prevented from reaccumulating radioiodide released by degradation of extrathyroidal organically bound iodine, the observed rate of decrease in thyroidal radioactivity should be a measure of thyroidal secretion. Therefore, during the administration of tapazole, thyroidal secretion of organic iodine was calculated as the product of the percentage rate of fall in thyroidal radioactivity and the thyroidal organic iodine pool.

*Calculation*—In R. D. (Figure 7) the thyroid lost 8.5 per cent per day of its initial activity for the first five days of tapazole therapy. Since the thyroid contained 8,450 micrograms of organic iodine at the onset of treatment, as calculated above, the rate of thyroidal secretion = 8.5 per cent per day × 8,450 micrograms = 720 micrograms per day.

Thyroidal secretion remained fairly constant for the first five to seven days of tapazole therapy but usually decreased thereafter in subjects with high rates of secretion (Figure 7, and J. St. in Figure 5). Values derived from the initial rates of secretion showed a range of 77 to 125 micrograms per day in euthyroid subjects and 450 to 925 micrograms per day in untreated hyperthyroid subjects (Table I).

The validity of these determinations is based on the assumption that the drug acts only to block thyroidal binding of iodine and has no direct effect on the rate of thyroidal secretion. The reasonable agreement between the values for thyroidal secretion obtained by this method and those calculated from the kinetics of distribution suggests that tapazole neither increases nor decreases the rate of secretion, at least initially. Further speculations regarding possible effects of tapazole on thyroid secretion are deferred to the Discussion.

An additional concern was whether an increase in the thyroidal *iodide* pool during tapazole administration was likely to introduce any error into the measurements of thyroidal organic $I^{131}$ and, therefore, into the calculations for thyroidal secretion. However, quantitative considerations indicate that in the absence of marked impairment of renal iodide excretion one may continue to regard all thyroidal radioactivity as organically bound without appreciable error.[4]

[4] This was experimentally confirmed in two subjects (P. G. and E. G.). In both cases 1.0 gm. sodium thiocyanate given intravenously after two and three weeks, respectively, of continuous tapazole administration failed

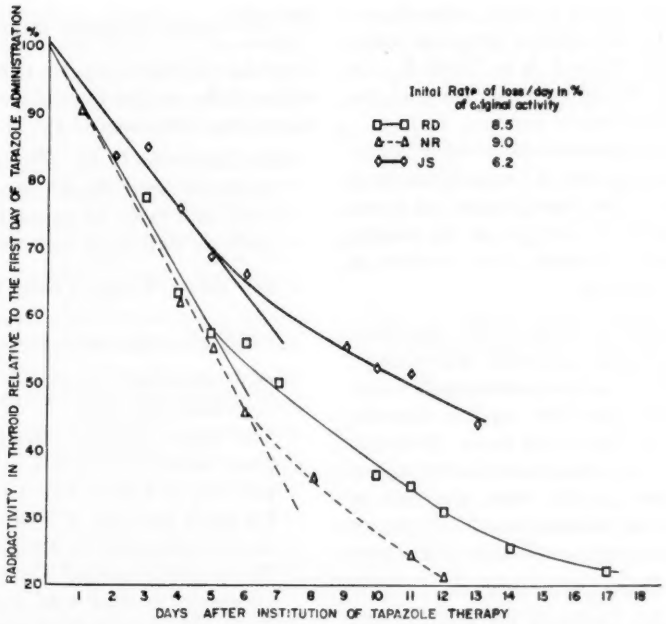


FIG. 7. RADIOACTIVITY IN THE THYROID DURING TAPAZOLE ADMINISTRATION

During the first five to seven days of therapy a constant amount of organic $I^{131}$ is secreted daily by the thyroid.

## PART III—METABOLIC DEGRADATION AND FECAL EXCRETION OF ORGANIC IODINE

### A. *Estimation of the rate of degradation from the renal "clearance" of plasma organic iodine*

#### 1. *In the presence of tapazole block*

Iodide released by metabolic degradation of extrathyroidal organic iodine is either excreted in the urine or reaccumulated by the thyroid. If thyroidal binding is completely blocked by large doses of tapazole, essentially all the iodide is excreted in the urine. Thus, although the kidneys do not directly clear the plasma of organic iodine to any significant extent, urinary iodide$^{131}$ under these conditions, represents clearance of metabolized plasma organic iodine (after the initially administered iodide$^{131}$ has disappeared from the circulation). This clearance has been termed "the apparent renal plasma organic iodine clearance" and is calculated from the daily (24-hour) urinary excretions of $I^{131}$ divided by the mean plasma concentration of $PBI^{131}$ over the 24-hour periods. The clearance rate is expressed as ml. plasma per day (Figure 3b), and is multiplied by the mean plasma $PBI^{127}$ concentration over the same period of time to give the rate of metabolic degradation of organic iodine$^{127}$ during tapazole administration.

*Calculation*—In R. D. (Figure 3b) the mean apparent renal plasma organic iodine clearance rate during the first few days of tapazole administration (12th to 16th days inclusive) averaged 3.60 liters per day. Since the plasma $PBI^{127}$ concentration at this time was 154 micrograms per liter, the rate of organic iodine degradation = 3.60 liters per day × 154 micrograms per liter = 555 micrograms per day.

to produce any detectable decrease in the residual thyroidal radioactivity over a two-hour period following the injections, demonstrating that there was no significant thyroidal iodide$^{131}$ at this time. To prove that this dose of sodium thiocyanate was sufficient to discharge thyroidal iodide when present, tracer tests with $I^{131}$ were performed one week later during tapazole inhibition of binding. In these studies all thyroidal radioactivity accumulated was discharged within a half hour by the same dose of thiocyanate.

The values for the rate of organic iodine degradation calculated by this method are given under the column headed Method A in Table I. In euthyroid subjects the rates ranged from 67 micrograms to 108 micrograms per day.

In addition to the determinations made immediately after the institution of tapazole therapy, such measurements were also obtained at intervals during the course of therapy, as the plasma PBI concentrations decreased, in a number of the hyperthyroid subjects.

*Calculation*—In R. D. (Figure 3b) the mean apparent renal plasma organic iodine clearance rate after about two and one-half weeks of tapazole therapy (28th to 31st days inclusive) was 2.1 liters per day. Since the plasma $PBI^{127}$ concentration was 69 micrograms per liter at this time, the rate of organic iodine degradation was 2.1 liters per day × 69 micrograms per liter = 144 micrograms per day.

Degradation rates obtained by this method during the course of tapazole induced remissions are plotted together with the initial determinations as a function of the $PBI^{127}$ concentrations in Figure 8. The degradation rates in hyperthyroid patients brought to normal PBI levels did not differ significantly from the values obtained in euthyroid subjects. In all hyperthyroid patients followed in this manner the "clearance" rates decreased strikingly as the plasma $PBI^{127}$ concentrations fell (Figure 3b).

2. *In the absence of tapazole block*

If reuptake of $iodide^{131}$ released by degradation of organic $iodine^{131}$ is not inhibited by tapazole or other agents, the radioiodide reaccumulated by the thyroid may be calculated from the urinary excretions of radioiodine and the $\frac{\text{thyroidal}}{\text{renal}}$ iodide accumulation ratio, assuming that all urinary $I^{131}$ is in the form of iodide. Neglecting objections to this assumption for the moment, the rate of metabolic degradation of organic iodine is then measured by the sum of the apparent renal and thyroidal plasma organic iodine clearances. Two determinations of the $\frac{\text{thyroidal}}{\text{renal}}$ iodide accumulation ratio were employed—the $\frac{\text{thyroidal}}{\text{renal}}$ iodide clearance ratio, and the ratio of thyroidal radioactivity to cumulative urinary radioactivity at the time of peak thyroidal uptake of the administered $I^{131}$ (Table I).

*Calculation*—In R. D. (Figure 3b) the mean apparent renal plasma organic iodine clearance rate prior to tapazole administration (7th to 10th days inclusive) was 1.0 liters per day. From Table I, the $\frac{\text{thyroidal}}{\text{renal}}$ iodide clearance ratio $= \frac{118}{34} = 3.46$ and the $\frac{\text{peak thyroidal uptake}}{\text{renal excretion}}$ ratio $= \frac{80}{26} = 3.08$. The mean apparent thyroidal plasma organic iodine clearance is, therefore, 1.0 liters per day × 3.46 = 3.46 liters per day and 1.0 liters per day × 3.08 = 3.08 liters per day, respectively, by each of these methods. The sum of thyroidal + renal organic iodine clearances is then 4.46 and 4.08 liters per day. When these figures are multiplied by the plasma $PBI^{127}$ concentration (154 micrograms per liter), values of 685 and 630 micrograms per day are obtained for organic iodine degradation.

Degradation rates calculated by these two methods are given under the columns headed Method B and Method C in Table I. Discrepancies between the results of each of these methods are simply a reflection of the experimental errors and biologic factors which make for inequality of the two iodide accumulation ratios. However, comparison of the values obtained by Methods B and C with those calculated by Method A must be viewed with a different significance. In most cases there was substantial agreement, indicating that prior to tapazole administration, urinary $I^{131}$ was almost entirely in a form which could be accumulated by the thyroid, presumably only iodide. In at least three hyperthyroid subjects (G. J., P. G., and W. R.), however, urinary $I^{131}$ during tapazole administration (Method A) was much lower than anticipated from the $\frac{\text{thyroidal}}{\text{renal}}$ iodide accumulation ratio (Methods B and C). The discrepancies appear too great to be accounted for on the basis of experimental error or incomplete inhibition by

tapazole. The implication, therefore, is that in the unblocked state of the gland in these subjects, some fraction of the urinary iodine was organically bound and not subject to thyroidal accumulation. In these cases the calculated plasma iodide levels prior to tapazole administration were the lowest among the patients studied (see Part IV). Thus, a relatively high proportion of organic $I^{131}$ in the urine may be partly related to a high $\frac{\text{PBI}}{\text{iodide}}$ ratio being presented to the kidneys. Quantitative evaluation of iodide and non-iodide fractions of urinary $I^{131}$ should permit reliable determinations of the organic iodine degradation rates by this method, in all cases, even in the absence of tapazole inhibition. During tapazole administration, most of the urinary $I^{131}$ represented iodide previously being accumulated by the thyroid and estimates of the organic iodine degradation rate were then not affected significantly by the presence of small amounts of organic $I^{131}$ in the urine.

In order to minimize errors resulting from changing blood levels and the lag in urinary excretion, the plasma organic iodine clearance measurements were made after the specific activity of the plasma had reached its peak and was changing at a relatively slow rate.[5] This procedure is also necessary to avoid including in the urinary excretions any iodide$^{131}$ remaining initially from the injected dose which had not been synthesized into an organically bound form by the thyroid; *e.g.*, note the higher excretions over the first few days shown in Figure 3.

B. *Estimation of the rate of degradation from the fraction $I^{131}_{ret}$ excreted in the urine and the total exchangeable organic iodine pool*

The daily organic iodine degradation is also given by the product of the fraction $I^{131}$ excreted in the urine daily during tapazole administration and the total exchangeable organic iodine pool, and may, likewise, be calculated from the urinary excretions prior to tapazole blockage and the $\frac{\text{thyroidal}}{\text{renal}}$ iodide accumulation. Since these determinations can be shown to be mathematically identical to those described in the previous paragraphs, they are not analyzed here in further detail.

C. *Rate of degradation of transfused exogenous biosynthesized organic iodine*

Since distribution of the labeled donor plasma iodine continued for a period of about 48 hours (Figure 6), the rate of degradation could be evaluated only from points taken subsequently. Unfortunately, collections of excreta were incomplete in these cases and so fecal excretion was estimated at 12½ per cent of degradation (see below). If it is assumed that urinary excretion of undegraded organic iodine was negligible and that in each case the recipient's organic iodine was metabolized at the same rate as the donor's, then the daily degradation rates were 256 micrograms, 68 micrograms, and 13 micrograms in the hyperthyroid, euthyroid and hypothyroid subjects, respectively (Table II).

Hamolsky, Freedberg, Kurland, and Wolsky (10) performed transfusion experiments similar to those presented here. However, these investigators followed the plasma curves for periods of only 23 to 70 hours and assumed that distribution was complete within 4 to 5 hours. In addition, no account was taken of fecal excretion of organic iodine. Their calculated degradation rates, therefore, are probably too high, and their spaces of distribution too low.

D. *The rate of organic iodine degradation as a function of PBI concentration in the plasma*

When the amount of organic iodine degraded daily was plotted against the plasma PBI$^{127}$ concentrations for all subjects, including the values obtained during periods of declining PBI concentrations under tapazole therapy (Figure 8), it became clear that degradation increases more rapidly than the plasma PBI concentration. The rate of organic iodine degradation was found to be roughly proportional to the square of the

[5] Since at average normal renal clearance rates, the half time for removal of iodide from the body by the kidneys is about nine hours (2) a brief calculation shows that under these conditions, following thyroid block, the quantity of radioactive iodine excreted in the urine is over 90 per cent on the second day and over 98 per cent thereafter of the amount undergoing degradation. This rapid equilibration between degradation and renal excretion has previously been demonstrated for the metabolism of $I^{131}$ labelled serum albumin (9). In the absence of thyroid inhibition, as is the case prior to tapazole administration, equilibration is even more rapid.

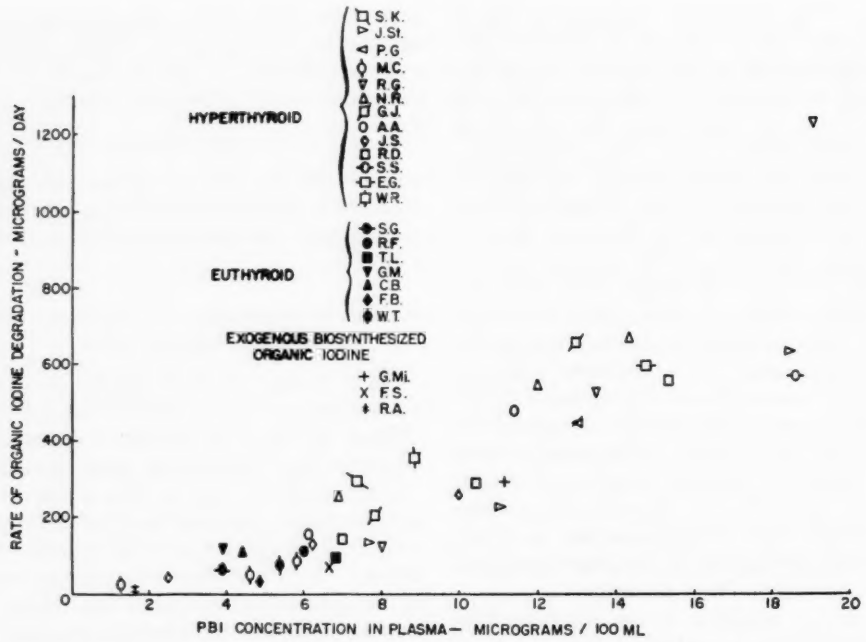


FIG. 8. RATE OF ORGANIC IODINE DEGRADATION AS A FUNCTION OF THE PBI CONCENTRATION IN THE PLASMA

The values for subjects R. D., R. G., N. R., M. C., J. S., A. A., and J. St., which are not included in Table I, were determined from the renal plasma organic iodine clearance rates during the course of tapazole administration as described in the text.

concentration (Figure 9) according to the following formula,

$$D = 2.94\ (PBI)^2,$$

where D = micrograms organic iodine degraded per day and (PBI) is in micrograms per 100 ml. plasma. This relationship is almost in exact agreement with that of Riggs (11) which was derived from data obtained during the administration of exogenous thyroid substance to hypothyroid and euthyroid subjects, and is in line with his suggestion that the metabolism of thyroid hormone is in the nature of a second order reaction.

The biologic life of biosynthesized organic iodine in extrathyroidal tissues is then an inverse function of the plasma concentration, with half times varying from about five days (14 per cent per day) at normal PBI concentrations to less than one and one-half days (50 per cent per day) at levels characteristic of marked hyperthyroidism.

E. *Fecal excretion of organic iodine*

For the first two or three days following the intravenous administration of $NaI^{131}$, fecal excretion usually was negligible indicating that iodide is not excreted in the stool to any significant extent. Following the appearance of $PBI^{131}$ in the plasma, the fecal excretion of organic iodine was calculated from the fecal organic iodine clearance rates.

*Calculation*—In R. D. (Figure 3b) the fecal organic iodine clearance rate averaged about 0.60 liters per day prior to the administration of tapazole. Since plasma PBI concentration was 154 micrograms per liter, fecal excretion = .60 liters per day × 154 micrograms per liter = 93 micrograms per day.

Fecal excretion of organic iodine generally amounted to about 10 to 15 per cent of the quantity degraded (Table I). However, there did not appear to be such a striking dependence of the fecal clearance rate on the plasma PBI concentrations as was observed with the degradation process. In hyperthyroid patients with decreasing PBI levels under the influence of tapazole therapy there was an occasional slight decrease in fecal clearance (Figure 3b). Euthyroid subjects appeared to have somewhat lower fecal clearance rates than the hyperthyroid patients but the data do not permit any definite expression of this relationship. In most of the subjects the rate of fecal clearance ranged from about 200 to 450 ml. per day. These values may be rederived from Table I by dividing the fecal excretions by the respective plasma PBI concentrations.

## PART IV—THE RATE OF REPLACEMENT OF THE ORGANIC IODINE POOL WITH NEWLY INGESTED IODINE; ENDOGENOUS AND EXOGENOUS IODINE BALANCE

### A. *Organic iodine replacement rate and thyroidal uptake of exogenous iodine*

In subjects who are in the steady state, synthesis of thyroid hormone from newly ingested food iodine (exogenous iodine) must replace that lost by excretion of previously synthesized organic iodine (endogenous iodine). Assuming steady state conditions, the organic iodine replacement rate may then be calculated as the sum of urinary and fecal excretions of endogenous iodine prior to tapazole administration divided by the total exchangeable organic iodine pool.

*Calculation*—In R. D. (Figure 3b) renal organic iodine clearance prior to tapazole administration was 1.0 liters per day. Since

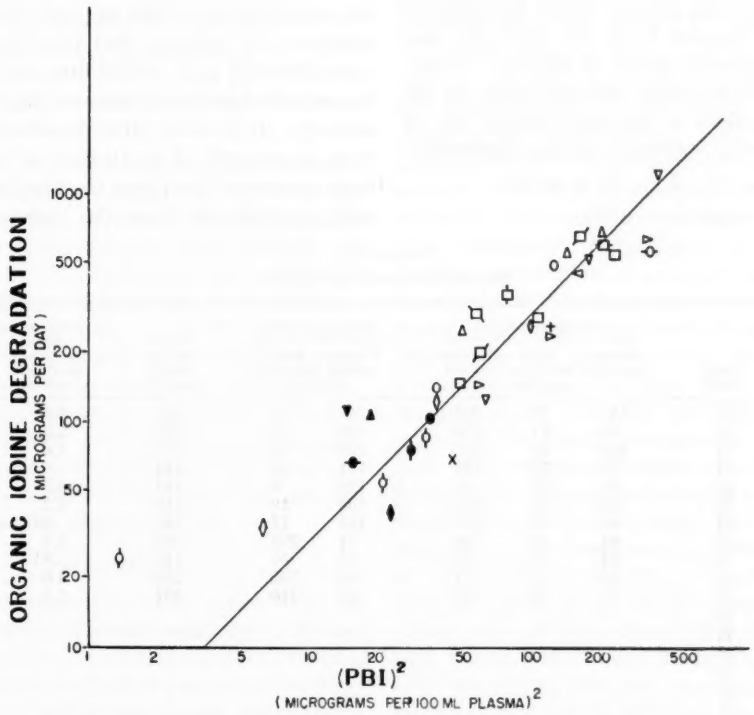


FIG. 9. LOG-LOG PLOT OF ORGANIC IODINE DEGRADATION AS A FUNCTION OF THE SQUARE OF THE PBI CONCENTRATION

The equation fitting the line is $D = 2.94\ (PBI)^2$.

the plasma PBI concentration was 154 micrograms per liter, the urinary excretion was 154 micrograms per day. Fecal excretion was 93 micrograms per day and, therefore, thyroidal uptake of exogenous iodine = 154 + 93 = 247 micrograms per day. Then, the fraction of the total pool (10,300 micrograms, as calculated above) replaced daily by exogenous iodine $= \frac{247}{10{,}300} = 2.4$ per cent per day.

Values for thyroidal uptake of exogenous iodine and the rate of organic iodine replacement are given in Table III. As expected, the replacement rates were higher in subjects with small pools and lower in those with large pools. Data are not given for subjects recently treated with tapazole since there is evidence to indicate that they may be in periods of positive iodine balance as a result of previous depletion of the thyroid glands.

Theoretically, the organic iodine replacement rate may be derived from the thyroidal and plasma radioactivity curves as follows. If thyroidal and extrathyroidal radioactivities are expressed as fractions of the administered dose of $I^{131}$, the following equations obtain (Appendix),

$$T = (T_p - c)e^{-\lambda_1 t} + ce^{-\lambda_2 t}$$

$$E = k(e^{-\lambda_1 t} - e^{-\lambda_2 t})$$

where T and E represent the fractions of the administered dose of $I^{131}$ in the respective compartments at any time, t, following peak uptake, $T_p$ is the fraction of the administered dose in the thyroid at peak uptake and $\lambda_2$ closely approximates the rate of exchange of newly ingested iodine with the total exchangeable organic iodine pool (organic iodine replacement rate). This is the rate at which the total exchangeable iodine pool would initially decrease, if iodine intake were abruptly discontinued and is clearly distinguished from the total turnover of organic iodine within the body which may be defined as the rate of thyroidal iodide accumulation or organic iodine secretion divided by the total exchangeable organic iodine pool. These equations are not very useful from the practical standpoint since the rate of organic iodine replacement (Table III), is usually too small (1 to 2 per cent per day) to permit accurate evaluation of this rate within the period of time limited by the rate of decay of the isotope. However, the equations do indicate that distribution in the extrathyroidal pool contributes significantly to the early biologic decay curve of thyroidal radioactivity. It is only after distribution equilibrium is attained (5 to 10 days in most of the cases presented here) that the loss from the thyroid parallels loss from the body and can be

TABLE III

*Derived data for iodine balance*

| Subject | Diag. No.* | Endogenous iodine | | | Exogenous iodine | | Total iodine intake | Organic iodine replacement rate | Calculated mean plasma iodide concentration |
|---|---|---|---|---|---|---|---|---|---|
| | | Renal excretion | Fecal excretion | Thyroid uptake | Thyroid uptake | Renal excretion | | | |
| | | μg./day | | | μg./day | | μg./day | %/day | μg./100 ml. |
| R. D. | H | 154 | 93 | 401 | 247 | 76 | 323 | 2.4 | .38 |
| J. St. | H | 88 | 81 | 556 | 169 | 27 | 196 | 1.9 | .22 |
| N. R. | H | 105 | 65 | 565 | 170 | 32 | 202 | 1.6 | .29 |
| S. K. | H | 75 | 26 | 221 | 101 | 40 | 141 | .52 | .19 |
| S. S. | H | 43 | 62 | — | 105 | 9 | 114 | 2.5 | — |
| P. G. | H | 67 | 41 | 378 | 108 | 10 | 118 | 1.2 | .10 |
| E. G. | H | 89 | 59 | 501 | 148 | 17 | 165 | .84 | .19 |
| T. L. | E | 81 | 13 | 26 | 94 | 291 | 385 | 3.9 | — |
| C. B. | E | 44 | 9 | 64 | 53 | 65 | 118 | .81 | .26 |
| W. T. | E | 55 | 5 | 21 | 60 | 180 | 240 | 5.0 | — |
| S. G. | E | 47 | 20 | 20 | 67 | 240 | 307 | 2.4 | .87 |
| A. A.† | H | | | | | | | | .68 |
| W. R.† | H | | | | | | | | .13 |
| R. G.† 1st study | H | | | | | | | | .53 |
| G. J.† 1st study | H | | | | | | | | .11 |
| G. M.† | E | | | | | | | | .38 |
| R. F.† | E | | | | | | | | .22 |

* H—Hyperthyroid.
E—Euthyroid.

† Fecal excretions were not obtained in these subjects. The plasma iodide concentrations were estimated by assuming that fecal excretion of organic iodine was 12½ per cent of the value obtained for organic iodine degradation.

taken as reflecting the rate of replacement of body organic iodine (Appendix). At this time the thyroidal and plasma curves should decrease exponentially with essentially the same slope.

B. *Estimation of iodine intake*

From the values for thyroidal uptake of exogenous iodine as derived above, the renal excretions of exogenous iodine were calculated, utilizing the $\frac{\text{renal}}{\text{thyroidal}}$ iodide accumulation ratios. When both ratios were available the mean value was utilized for these calculations. Since fecal excretion of iodide is negligible, the iodine intake is then given by the sum of exogenous iodine accumulated by the thyroid and that excreted in the urine. In order to complete the balance sheet, thyroid uptake of endogenous iodine (Table III) is calculated from the increase in renal excretion of radioiodine following tapazole administration or from the renal excretion and the $\frac{\text{thyroidal}}{\text{renal}}$ iodide accumulation ratio in cases where tapazole was not administered.

C. *Estimation of mean plasma iodide concentrations*

Since daily thyroidal accumulation (endogenous plus exogenous iodine) is equal to the product of the daily thyroidal clearance of iodide and the mean plasma iodide concentration, the latter could be calculated from the other data available. The calculated plasma iodide concentrations ranged from 0.1 to 0.5 microgram per liter plasma in all but two subjects for whom sufficient data were available (Table III). There appeared to be no consistent differences between euthyroid and hyperthyroid subjects, except that the lowest calculated iodide concentrations were obtained in patients with markedly elevated thyroidal clearance rates.

## DISCUSSION

The studies presented give no insight into the qualitative aspects of iodine metabolism. Future investigations into the relative roles of thyroxine, tri-iodo-thyronine and other possible physiologically important iodine compounds undoubtedly will modify the meaning of any quantitative data regarding total iodine metabolism. However, the parallel relationship between thyroidal activity, tissue metabolism and clinical state, on one hand and total thyroidal iodine accumulation and release and peripheral organic iodine degradation on the other, give significance to such data even in the absence of information regarding the specific hormonally active substances. Results obtained in the cases studied indicate that this parallel relationship obtains in general, regardless of the pathological state of the thyroid gland. There is no evidence to suggest any peripheral abnormality in patients with Graves' disease since so-called "utilization" of "hormonal" iodine appeared to be related primarily to the concentration of circulating organic iodine.

*Mixing of the $I^{131}$ tracer in the thyroidal organic iodine pool*

Since there is some histologic evidence for variability of functional activity in different areas of the thyroid, it is perhaps somewhat surprising that the radioiodine tracer appears to mix so uniformly with the thyroidal pool. However, the maintenance of horizontal plateaus in the plasma concentration curves (fraction $I^{131}_{rel}$) for two to three weeks prior to tapazole and the constancy of the plasma specific activity even after depletion of 80 to 90 per cent of thyroidal radioactivity under tapazole therapy leave little doubt as to the uniformity of distribution of the $I^{131}$ in a "functional" thyroid pool in these cases. Further confirmation is provided by the general agreement between thyroidal secretion following tapazole block and the sum of organic iodine degradation and excretion. On the other hand, it must be suspected that, not infrequently, labelling of the thyroidal pool with radioactive iodine may be much less uniform, especially in cases of functioning adenomata and in glands of low activity. Such may actually have been the situation in G. M., the only case in which a plateau in the plasma was not reached during the period of observations. If uniform mixing is not obtained, then measurements of the thyroidal pool and the rate of thyroidal secretion after tapazole block may be inaccurate. However, even if so, the lack of reliability of these measurements has no influence whatever on the validity of the calculations for organic iodine degradation, fecal excretion, extrathyroidal space of organic iodine distribution and the size of the

extrathyroidal pool, all of which are independent of uniformity of mixing of the tracer in the thyroidal pool.

*Concentration of PBI$^{131}$ in the plasma as a diagnostic aid*

Numerous studies have employed the plasma concentration of PBI$^{131}$ as a measure of thyroidal function. The plasma concentration of PBI$^{131}$ is influenced by the following factors: The fraction of the administered dose accumulated by the thyroid (which is determined by the ratio of thyroidal to renal iodide clearance), the rate of secretion of organically bound iodine by the thyroid, the size of the thyroidal organic iodine pool, the extrathyroidal space of distribution of organic iodine, the rates of peripheral metabolism and fecal excretion and the time following administration. For any early time the three most important factors are the fraction of the dose accumulated, the size of the thyroidal pool, and the rate of secretion from the thyroid. The first of these is affected by the level of renal clearance and is, therefore, sensitive to an extrathyroidal influence. Moreover, under certain circumstances, changes in the size of the thyroidal pool may so outweigh the effect of the other factors as to result in disparately high or low values for PBI$^{131}$. Blom and Terpstra (12) and Ingbar, Freinkel, Hoeprich, and Athens (13) have observed cases of post-thyroidectomy myxedema with high PBI$^{131}$ concentrations in the plasma and have interpreted this as resulting from a small thyroidal pool. In the present study most of the euthyroid subjects previously treated for hyperthyroidism by surgical or radioiodine ablation of the thyroid had small thyroidal pools and high plasma concentrations of PBI$^{131}$, the latter reaching levels of 0.4 per cent of the administered dose per liter plasma on the third or fourth day. In patients with thyroid toxicity persisting after I$^{131}$ therapy, Chapman, Maloof, Maisterrena, and Martin (14) were puzzled to explain 48-hour thyroidal uptakes of only about 48 per cent. It is reasonable to suggest that the I$^{131}$ therapy had reduced the size of the functional thyroidal pool so that at 48 hours the thyroidal uptakes were significantly below the peak uptake values as a result of secretion into the extrathyroidal pool. Even in untreated cases of hyperthyroidism a fall in thyroidal radioactivity of 10 to 15 per cent from peak uptake to the 48-hour point is not uncommon. An example of the opposite situation is presented by S. K., a hyperthyroid subject with a thyroidal pool of almost 20,000 micrograms iodine who showed a *peak* concentration in the plasma of only 0.15 per cent of the administered dose per liter and less than 0.1 per cent on the third day. It is clear that the diagnosis of relapse of hyperthyroidism after therapy of Graves' disease should not be influenced by high plasma PBI$^{131}$ concentrations and also that occasional cases of hyperthyroidism with large thyroidal iodine pools may fail to show high PBI$^{131}$ concentrations in the plasma.

*Effect of tapazole on thyroid secretion*

The nature of the factors controlling iodine release from the thyroid during tapazole administration is not clear. For the first few days, release did not appear to be affected as judged by comparison with the values obtained from the distribution curves prior to tapazole blockage, and those determined for the sum of peripheral degradation and fecal excretion at the same time. In hyperthyroid subjects, thyroidal secretion is definitely retarded after several days of tapazole therapy but this may be related to the decrease in the organic iodine content of the thyroid rather than to a specific effect of tapazole. In view of the falling plasma PBI levels, the decrease in the rate of release of thyroidal iodine is unlikely to be due to diminished pituitary secretion of TSH. Perhaps the thyroid itself is sensitive to changes in its hormonal iodine content and acts to prevent a rapid depletion of iodine stores. An alternative possibility is that an increase in the thyroidal iodide pool, resulting from inhibition of binding, may directly or indirectly effect a decrease in the rate of release of organic iodine by the thyroid. This would be consistent with the effect of iodine administration which also retards release of hormone and yet increases both the organic iodine and iodide contents of the thyroid, at least temporarily. The maintenance of low thyroidal iodide concentrations with thiocyanate or similar agents may aid in the evaluation of the alternative possibilities.

These studies do not confirm Riggs' suggestion (11) that tapazole may *increase* discharge from the thyroid. For in that event either the plasma PBI (I$^{127}$ and I$^{131}$) levels should have risen or the

rate of degradation should have increased over that prior to the administration of tapazole. No significant changes in these directions were ever observed. Furthermore, the values for thyroidal secretion during the first few days of tapazole administration agreed quite reasonably with those obtained independently for the sum of organic iodine degradation and fecal loss. Had tapazole accelerated the rate of thyroidal secretion significant discrepancies should have been observed.

*Therapeutic implications*

Since seven to nine per cent of the total iodine pool is degraded daily in some of the hyperthyroid patients, it is evident that complete blockage of iodine accumulation by the thyroid must soon lead either to depletion of available hormone stores or to a significant reduction in the rate of organic iodine degradation and, therefore, to the rapid remission of clinical symptoms. This serves to explain why the most toxic patients often exhibit the most dramatic clinical response to this type of high dosage therapy. The lower dose levels commonly employed in the treatment of hyperthyroidism generally permit thyroidal reaccumulation of 40 per cent or more of iodide released by degradation of organic iodine, as indicated by $I^{131}$ tracer studies (4). Under these conditions the iodine stores are depleted much less rapidly. Since patients treated with the higher doses (25 mg. every six hours) do not exhibit an increased frequency of toxic reactions, it would seem advisable to institute therapy with completely inhibiting doses and to reduce the dosage only after a euthyroid state has been attained.

## SUMMARY AND CONCLUSIONS

1. Methods are presented for the determination of the exchangeable organic iodine pool and the rates of thyroidal secretion, peripheral degradation, and fecal excretion of endogenously synthesized organically bound iodine. These determinations require only assays of radioactivity following the administration of $I^{131}$ and measurements of the protein bound iodine concentration in the plasma.

The distribution and degradation of exogenously synthesized organically bound iodine were studied in three subjects.

2. The exchangeable thyroidal pool of organic iodine does not differ markedly in untreated hyperthyroidism and euthyroidism but may be strikingly reduced following ablation therapy or treatment with tapazole. The apparent extrathyroidal space of distribution of biosynthesized organic iodine averages about 9 liters or 10 to 20 per cent of body weight.

3. At normal serum PBI levels, approximately 40 to 110 micrograms of iodine are degraded daily in euthyroid subjects and in patients with hyperthyroidism maintained in a euthyroid state clinically by treatment with tapazole. The rate of degradation increases with the square of the PBI concentration over a greater than five-fold range in concentration. There was good agreement between the values obtained for thyroidal secretion and the sum of degradation plus fecal loss.

4. Fecal excretion of organic iodine amounts to approximately 10 to 15 per cent of the quantity degraded in all subjects.

5. Data for exogenous and endogenous iodine balance are derived from the assumption of steady state conditions.

6. The kinetics of distribution and degradation of organic iodine are analyzed and discussed with particular reference to the significance of plasma $PBI^{131}$ concentrations and the characteristics of the biologic decay curve of thyroidal radioactivity.

7. Certain therapeutic implications of these studies are considered.

## APPENDIX

Let T and E represent the fractions of the administered dose of $I^{131}$ in thyroidal and extrathyroidal compartments at any time, t, following peak uptake by the thyroid, $\lambda_{TE}$ and $\lambda_{ET}$ be the rate constants of transfer between the two compartments respectively in the order of the subscript designations and $\lambda_{EU}$ and $\lambda_{EF}$ be the rate constants of transfer from the extrathyroidal compartments to urinary and fecal excretions respectively. Let $\lambda_{EO} = \lambda_{EU} + \lambda_{EF}$. Then,

$$\frac{dT}{dt} = -\lambda_{TE}T + \lambda_{ET}E \qquad (1)$$

$$\frac{dE}{dt} = \lambda_{TE}T - \lambda_{ET}E - \lambda_{EO}E \qquad (2)$$

Utilizing the conditions,

at $t = 0$, $E = 0$ and $T = T_p$

$= $ peak uptake by the thyroid,

the equations are solved for T and E,

$$T = (T_p - c)e^{-\lambda_1 t} + ce^{-\lambda_2 t} \qquad (3)$$

$$E = k(e^{-\lambda_1 t} - e^{-\lambda_2 t}) \qquad (4)$$

where

$$\lambda_1 = \tfrac{1}{2}[(\lambda_{TE} + \lambda_{ET} + \lambda_{EO}) - \{(\lambda_{TE} + \lambda_{ET} + \lambda_{EO})^2 - 4\lambda_{TE}\lambda_{EO}\}^{\frac{1}{2}}] \quad (5)$$

$$\lambda_2 = \tfrac{1}{2}[(\lambda_{TE} + \lambda_{ET} + \lambda_{EO}) + \{(\lambda_{TE} + \lambda_{ET} + \lambda_{EO})^2 - 4\lambda_{TE}\lambda_{EO}\}^{\frac{1}{2}}] \quad (6)$$

$$c = \frac{T_p(\lambda_{TE} - \lambda_1)}{\lambda_2 - \lambda_1} \quad (7)$$

$$k = \frac{\lambda_{TE}T_p}{\lambda_2 - \lambda_1} \quad (8)$$

$\lambda_2$ and $\lambda_1$ will differ maximally as the value of the discriminant in (5) and (6) approaches $\lambda_{TE} + \lambda_{ET} + \lambda_{EO}$. This is approximated when

$$(\lambda_{TE} + \lambda_{ET} + \lambda_{EO})^2 \gg 4\lambda_{TE}\lambda_{EO}$$

or

$$\lambda_{TE}^2 + \lambda_{ET}^2 + \lambda_{EO}^2 + 2\lambda_{ET}\lambda_{EO} + 2\lambda_{TE}\lambda_{ET} \gg 2\lambda_{TE}\lambda_{EO}$$

In hyperthyroidism $\lambda_{ET}$ is two to three times $\lambda_{EO}$ and $\lambda_{ET}$ is generally five to ten times $\lambda_{TE}$. The inequality is then seen to be satisfied. In most cases of euthyroidism where the thyroidal pool is much greater than the extrathyroidal pool, then $\lambda_{ET} \gg \lambda_{TE}$ and the inequality is also satisfied. However, in certain cases in which the thyroidal pool is small compared to the extrathyroidal pool, due to ablation therapy or other cause, and in which $\lambda_{EO}$ is large compared to $\lambda_{ET}$, the inequality is seen to be less well satisfied. Therefore in all cases except the last, $\lambda_1$ is small compared to $\lambda_2$.

The organic iodine replacement rate (R) is defined as

$$R = \frac{\lambda_{EO} \times \text{extrathyroidal pool}}{\text{Total exchangeable pool}} = \frac{\lambda_{EO}E_{pool}}{E_{pool} + T_{pool}}$$

where $E_{pool}$ and $T_{pool}$ are the quantities of organic iodine$^{127}$ in the respective pools. Since $\lambda_{TE}T_{pool} = (\lambda_{EO} + \lambda_{ET})E_{pool}$, then

$$R = \frac{\lambda_{EO}\lambda_{TE}}{\lambda_{TE} + \lambda_{ET} + \lambda_{EO}} \quad (9)$$

The purpose of the following section is to demonstrate that when $\lambda_1$ is small compared to $\lambda_2$, $\lambda_1$ approximates R.

Equation (5) can be rewritten in the form

$$\lambda_1 = \tfrac{1}{2}\left[(\lambda_{TE} + \lambda_{ET} + \lambda_{EO}) - (\lambda_{ET} + \lambda_{TE} + \lambda_{EO}) \times \left(1 - \frac{4\lambda_{TE}\lambda_{EO}}{(\lambda_{TE} + \lambda_{ET} + \lambda_{EO})^2}\right)^{\frac{1}{2}}\right] \quad (10)$$

As shown above, $\lambda_1$ is small compared to $\lambda_2$ when

$$\frac{4\lambda_{TE}\lambda_{EO}}{(\lambda_{TE} + \lambda_{ET} + \lambda_{EO})^2} \ll 1$$

Then,

$$\left[1 - \frac{4\lambda_{TE}\lambda_{EO}}{(\lambda_{TE} + \lambda_{ET} + \lambda_{EO})^2}\right]^{\frac{1}{2}} \cong 1 - \frac{2\lambda_{TE}\lambda_{EO}}{(\lambda_{TE} + \lambda_{ET} + \lambda_{EO})^2}$$ [6]

Substituting in (10) and (9),

$$\lambda_1 \cong \frac{\lambda_{TE}\lambda_{EO}}{\lambda_{TE} + \lambda_{ET} + \lambda_{EO}} = R$$

[6] This is easily recognized as the approximation $(1 + x)^{\frac{1}{2}} \cong 1 + \frac{x}{2}$ when $x \ll 1$.

ACKNOWLEDGMENTS

We are greatly indebted to Dr. F. R. Keating, Jr. and Dr. N. B. Myant for critically reviewing the manuscript. We also wish to thank Mr. John Hessian and Mr. Paul Newman for the illustrations and Mrs. Frieda Steiner and Miss Eve Spelke for secretarial assistance.

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# PROFIBRINOLYSIN, ANTIFIBRINOLYSIN, FIBRINOGEN AND URINE FIBRINOLYTIC FACTORS IN THE HUMAN SUBJECT [1]

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(Submitted for publication March 30, 1954; accepted July 22, 1954)

This report describes methods for the quantitative assay of human plasma profibrinolysin (plasminogen) and for the semi-quantitation of fibrinolytic factors in urine. The concentrations of plasma profibrinolysin, antifibrinolysin, fibrinogen and lytic factors in urine of patients with an active neoplasm and in pregnant women are compared with their concentrations in normal individuals.

Although several methods have been described for the separation of human profibrinolysin from other constituents of plasma (1–5), the method described here appears to have the advantages that: (a) It can be utilized for quantitation; (b) it can be performed on a relatively small aliquot of plasma; (c) it is not influenced by the antifibrinolysin titer of the plasma; (d) it is highly reproducible; and (e) it does not require complex procedures or reagents which are difficult to obtain.

The blood and urine of approximately 200 human subjects were analyzed. Significant mean increases in plasma profibrinolysin, antifibrinolysin, and fibrinogen were found during pregnancy and in patients with neoplastic disease when compared with normal subjects. The fibrinolytic activity of urine was slightly but not significantly higher in patients with neoplasms than in normal individuals.

## MATERIALS AND METHODS

*Reagents.* Protamine sulfate powder (Salmine) [2] was dissolved in 0.9 per cent NaCl to a final concentration of 0.8 per cent.

Imidazole, employed to buffer the fibrin clots at pH 7.25, was prepared by dissolving 1.72 g. of the free base in 90 ml. of 0.1 M HCl. The pH was adjusted to 7.25 with HCl or NaOH, and the volume brought to 100 ml. with distilled, or demineralized, water.

Streptokinase (Varidase®) [3] was used to activate the profibrinolysin to fibrinolysin. The streptokinase-streptodornase preparation was dissolved in 0.9 per cent NaCl solution to produce a streptokinase solution containing 15,000 units per ml.

The fibrinogen used for preparation of the fibrin substrate was obtained from bovine plasma by the freeze-thawing method (6). A 1.5 to 3.0 per cent fibrinogen solution containing 1.5 per cent NaCl and 5 per cent of the imidazole buffer solution was stored at −20° C. Prior to use, a test tube containing the stored fibrinogen solution was thawed in a 37.5° C. water bath. The fibrinogen was then diluted with 0.9 per cent NaCl and distilled water to a fibrinogen concentration of 0.2 per cent and a final salinity equivalent to 0.9 per cent NaCl. These fibrinogen solutions were stable for five hours or longer when maintained at 37.5° C.

Thrombin, Topical,[4] was employed to clot the fibrinogen. The solution, containing 2500 Iowa units per ml., was prepared by dissolving the thrombin in 0.9 per cent NaCl and adding 50 per cent glycerol for stabilization during storage under refrigeration.

Bovine fibrinolysin [5] was dissolved and standardized for the antifibrinolysin assay as described in previous reports (7, 8).

The phenol reagent of Folin and Ciocalteu (9) was used to determine tyrosine in the assay of fibrinogen.

*Collection and preparation of plasma.* Nine parts of human blood, obtained from the antecubital vein without stasis, was immediately mixed with one part of 3.2 per cent sodium citrate. The plasma was removed by pipetting, after centrifugation and reading of the hematocrit. All fibrinogen determinations were completed during the day on which the blood was drawn. The plasma was stored at −20° C. whenever profibrinolysin or antifibrinolysin assays could not be completed on the same day.

[1] This work was supported by a Cancer Control Grant from the United States Public Health Service.

[2] Supplied through the kind cooperation of Dr. W. R. Kirtley of Eli Lilly and Co.

[3] Generously supplied by Dr. J. M. Ruegsegger of the Lederle Laboratories Division, the American Cyanamide Co.

[4] Generously supplied through the courtesy of E. C. Loomis of Parke, Davis and Co.

[5] Generously supplied through the courtesy of E. C. Loomis of Parke, Davis and Co.

*Profibrinolysin isolation and assay.* The separation of profibrinolysin from antifibrinolysin and other interfering substances was accomplished by treatment with protamine sulfate, alteration in pH, dilution, centrifugation, and digestion of any remaining fibrinogen following activation of the proenzyme with streptokinase. Studies have indicated that this procedure results in the quantitative precipitation of profibrinolysin. Four mg. of protamine sulfate were added to a 2 ml. aliquot of the plasma by pipetting in 0.5 ml. of the protamine sulfate solution. The pH of the mixture was brought to 9.0 (glass electrode) by adding 0.1 M NaOH dropwise with stirring. After allowing the solution to remain at pH 9.0 for one hour at room temperature, it was diluted with distilled or demineralized water to a volume of 40 ml. and the pH adjusted to 7.0 (glass electrode) with 0.1 M HCl. With dilution and lowering the pH a fairly heavy precipitate, which contained fibrinogen and profibrinolysin, appeared. The precipitate was separated in 50 ml. containers by centrifugation for 30 minutes at 3000 r.p.m. (2000 RCF). The supernatant, containing antifibrinolysin, was decanted and discarded. No measurable fibrinolytic activity following treatment with streptokinase has ever been found in numerous tests of the supernatant. Traces of antifibrinolysin occasionally remained with the precipitate, but the amount present was barely detectable by the most sensitive assay methods. The trace contamination with antifibrinolysin was removed by repeated washing with distilled water and recentrifugation in several experiments but this procedure did not improve the accuracy or precision of the method.

When added to the test plasma, profibrinolysin preparations prepared by the method of Kline (10), may be recovered as fibrinolysin. However, if an appreciable quantity of profibrinolysin is added to plasma, the amount of streptokinase utilized for activation must also be increased, since the reaction between streptokinase and profibrinolysin appears to be stoichiometric (11). In the analyses reported here, additional streptokinase was added to the precipitates from a number of the native plasmas in which the fibrinolytic activity, as measured in the conventional assay, was high. No additional fibrinolysin was recovered when the higher concentrations of streptokinase were used, indicating that the streptokinase preparations in use for the assays reported here were adequate for complete activation. Furthermore, the statistical increase obtained in profibrinolysin in malignancy and pregnancy is indicative of adequate activation of the proenzyme.

No fibrinolytic activity was detectable in the precipitates. To activate the proenzyme, the precipitate was brought to a volume of approximately 2 ml. by suspending it in a solution containing 0.7 ml. imidazole buffer, 1.0 ml. of 0.9 per cent NaCl and 0.1 ml. of the streptokinase solution (1500 units). During an incubation period of one-half hour in the 37.5° C. water bath the profibrinolysin was converted to fibrinolysin and the fibrinogen residues which remained in the precipitate were digested.

In determining the fibrinolytic activity, aliquots of the activated re-dissolved precipitate were diluted with saline to adjust the lysis times of 0.1 per cent fibrin clots to between 200 and 400 seconds.[6] Dilutions of the fibrinolysin in the clot between 1 to 16 and 1 to 64 usually produced lysis within the prescribed time range. In making this assay, 0.2 ml. of the diluted, activated fibrinolysin solution to be tested was pipetted into a test tube, 50 mm. × 8 mm. I.D. As 0.2 ml. of 0.2 per cent fibrinogen was pipetted into the tube a stop watch was started. Thrombin was immediately added by stirring the mixture with a 2 mm. diameter rod which had been wetted with the thrombin described above. The tube was placed in a 37.5° water bath and allowed to remain there except when examined for the lytic end-point. A solid clot formed within 15 to 20 seconds after the thrombin was added. The progress of the lysis was followed by tipping the tube at intervals. Just prior to the end-point the clot acquired a semifluid consistency and slid down the tube with a humped appearance. The end-point was reached when the "hump" disappeared. Occasional debris was disregarded. Lysis times were determined in triplicate for each 2 ml. sample of plasma and in most cases duplicate samples of plasma were analyzed.

The 37.5° C. assay curve in Figure 1 was based upon dilutions of the activated plasma profibrinolysin (procedure described above) from approximately 200 individuals. The average values for each dilution of all determinations were utilized in constructing this curve. This 37.5° curve coincided with the curve obtained by dilution of bovine fibrinolysin (Loomis) with assay at 37.5° C. and the units are the same as those which have previously been described except that the centiunit is 1/100 of the conventional unit. The 28° C. curve is included since the assay for fibrinolysin was originally described at this temperature and repeated checks have indicated that the human profibrinolysin assay also falls on this curve when the lysis is carried out at 28° C. The identical slopes of the human profibrinolysin assay curves and the bovine fibrinolysin assay curves would indicate that there is no more inhibitor in our profibrinolysin preparations than in the best bovine fibrinolysin available.

*Antifibrinolysin assay.* This assay has been described in detail (7, 8); in essence it involves measuring the amount of residual bovine fibrinolysin, following incubation under controlled conditions of a known quantity of fibrinolysin with diluted defibrinated plasma. Arbi-

[6] If the dilutions do not follow the assay curves (Figure 1) when clots lyse within the 200 to 400 seconds time range, greater dilutions of the material (longer lysis times) fall on the curve. In all of the studies reported in this paper, dilutions followed the curve when the lysis times were between 200 and 400 seconds. However, with some streptokinase preparations which have been obtained recently the dilutions did not follow the curve in the higher concentration ranges. This discrepancy appears to result from inhibitors associated with the streptokinase.

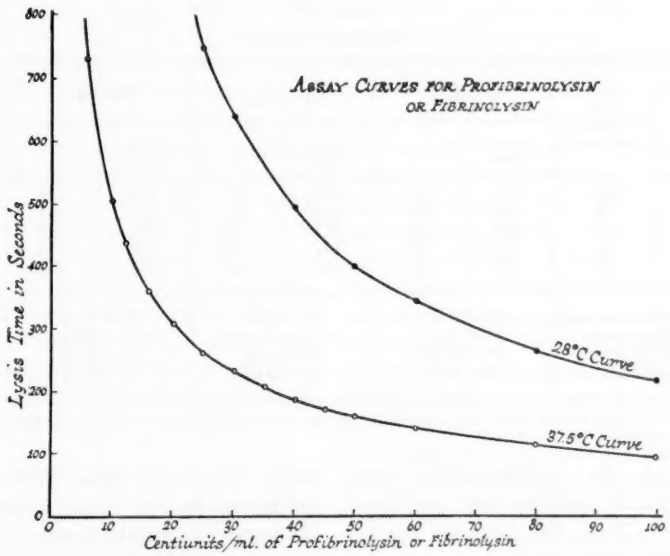


FIG. 1. ASSAY CURVES FOR HUMAN PROFIBRINOLYSIN OR FIBRINOLYSIN AT 28° C. AND 37.5° C., BASED UPON THE AVERAGE LYSIS TIMES AT EIGHT DILUTIONS OF 200 HUMAN PLASMA FIBRINOLYSIN PREPARATIONS, EACH PREPARED IN DUPLICATE

These curves coincide with curves at 28° C. and 37.5° C. prepared by dilution of bovine fibrinolysin supplied by E. C. Loomis.

trary units of antifibrinolysin are based on the quantity of fibrinolysin inactivated.

*Fibrinogen assay.* The method of Ware, Guest, and Seegers (6) was employed, in which the fibrinogen in diluted plasma is clotted with thrombin; the fibrin clot is wound out on a stainless steel wire, washed with saline and hydrolyzed by NaOH at 100° C. The liberated tyrosine is determined by the method of Folin and Ciocalteu (9).

*Assay of urine fibrinolytic factors.* In this study no attempt was made to differentiate between urine kinase, an enzyme which accelerates the conversion of profibrinolysin to fibrinolysin, and urine fibrinoprotease, an enzyme which causes direct lysis of the fibrin clot in the absence of profibrinolysin.

Assay for the fibrinolytic factors was carried out on the first morning specimen from normal individuals and all patients except pregnant women. The urine was dialyzed against running tap water until a specific resistance of over 7000 ohms was obtained. A 0.3 ml. aliquot of the dialyzed urine was placed in the same type test tube used in the profibrinolysin and antifibrinolysin assays. To this was added 0.1 ml. of 0.2 per cent fibrinogen solution and the mixture clotted by the addition of thrombin with a stirring rod. The tubes were placed in a 37.5° water bath until the lysis end-point described above was obtained.

## RESULTS

The maximum variation in duplicate profibrinolysin determinations on the same blood sample was ± 10 per cent of the mean of the two values. The range in variation of profibrinolysin titers between individuals was not as great as for fibrinogen and antifibrinolysin. The lowest titer encountered was 91 centiunits in a normal individual and the highest was 260 centiunits in a patient with a breast carcinoma. However, there was a tendency toward an increase in profibrinolysin in plasmas from patients with neoplasms and in plasmas from pregnant women when compared with the mean concentration in plasmas from normal individuals. The results are graphically summarized in Figure 2 and the statistical significance of the differences is indicated in Table I.

The lowest antifibrinolysin titer was 57 units in a normal individual and the highest was 376 units in a patient with carcinoma of the breast. Pregnant women as a group, however, exhibited the highest antifibrinolysin titers of the categories

TABLE I *

*Statistical analysis*

| | No. plasma samples analyzed | Pro-fibrinolysin | | Anti-fibrinolysin | | Fibrinogen | | Urine lysis | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean units | σ | Mean units | σ | Mean mg. % | σ | Mean times | σ |
| Normal | 26 | 154 | 25 | 110 | 27 | 268 | 72 | 37 | 13 |
| All neoplasms | 74 | 174 | 43 | 136 | 47 | 352 | 92 | 32 | 12 |
| Skin tumors | 29 | 180 | 45 | 117 | 32 | 351 | 88 | 34 | 15 |
| Ca breast | 20 | 181 | 50 | 147 | 65 | 330 | 57 | 32 | 12 |
| Pregnancy | 24 | 191 | 21 | 207 | 36 | 584 | 134 | — | — |
| All neoplasms higher than normal | | $P < 0.01$ | | $P < 0.01$ | | $P < 0.01$ | | — | |
| Skin tumor higher than normal | | $P < 0.01$ | | not significant | | $P < 0.01$ | | — | |
| Ca breast higher than normal | | $P < 0.03$ | | $P < 0.02$ | | $P < 0.01$ | | — | |
| Pregnancy higher than normal | | $P < 0.01$ | | $P < 0.01$ | | $P < 0.01$ | | — | |

* Probability, $P < 0.01$, means that the probability of the result being due to chance is less than 1 in 100. The probability, P, was derived from the t test.

studied. Although the antifibrinolysin titers tended to be higher in patients with neoplasms than in normal individuals, this alteration was too variable to be of diagnostic significance.

The tendency for an increase in concentration of a specific plasma protein in disease and in pregnancy was of the same general magnitude for fibrinogen as for antifibrinolysin. The lowest fibrinogen concentration was 153 mg. per cent in a normal individual and the highest was 859 mg. per cent in a pregnant patient. Thus the highest fibrinogen concentration was 450 per cent greater

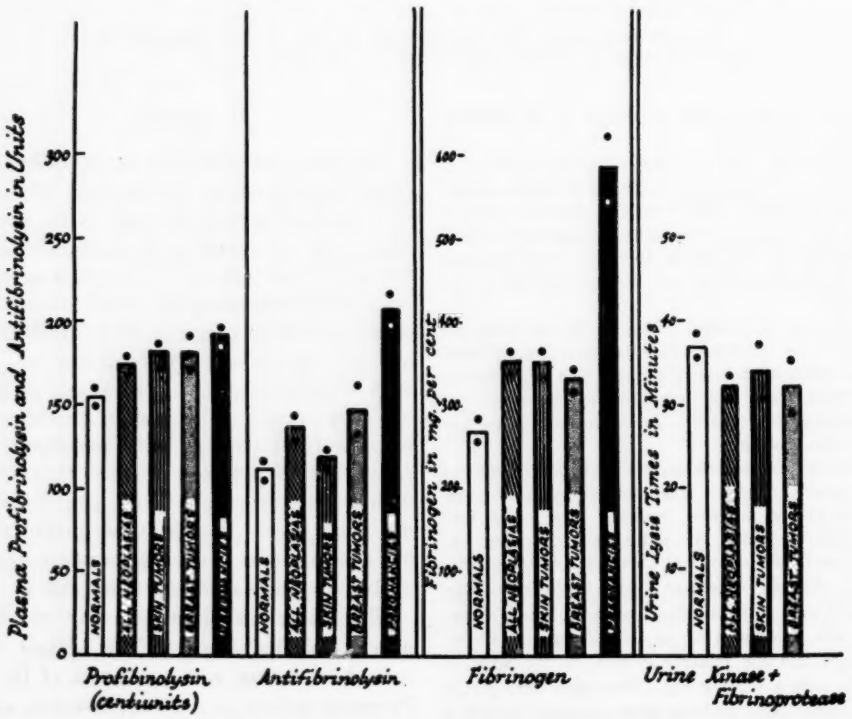


FIG. 2. GRAPHICAL SUMMARY OF DATA ON NORMAL INDIVIDUALS AND HOSPITAL PATIENTS

than the lowest fibrinogen concentration in this series and the highest antifibrinolysin titer was 560 per cent greater than the lowest. In contrast the highest profibrinolysin titer was only 94 per cent greater than the lowest. Again, as with antifibrinolysin titers, pregnant women exhibited the highest fibrinogen concentrations of any group.

Lysis of 0.05 per cent fibrin clots by the urine dialysates from different individuals required from 12 to 75 minutes. Lysis times observed with the dialyzed urine from patients with neoplasms tended to be shorter than those found in dialyzed urine of normal individuals. However, as with the other determinations there was considerable overlap among individuals of the pathological and normal groups.

## DISCUSSION

A large gap in our present knowledge of the profibrinolysin - fibrinolysin - antifibrinolysin system involves the means by which profibrinolysin is physiologically activated to fibrinolysin. Although kinases from various tissues and physiologic fluids have been described, their functional significance is unknown. Astrup and Permin (12), Permin (13), Tagnon and Petermann (14), and Lewis and Ferguson (15), have obtained factors from tissues which accelerate the conversion of profibrinolysin to fibrinolysin, and Lewis and Ferguson have reported that a fibrinokinase is present in canine serum (16). A fibrinokinase has also been found in urine (17–19). However, it has not been demonstrated that any of these factors are responsible for the modification of the profibrinolysin-fibrinolysin-antifibrinolysin system which has been reported in conditions of shock (20), general stress (21, 22) or allergic states (5, 22).

Since the physiologic mechanism of activation of profibrinolysin is unknown and physiologic activators are not now available, it is necessary for assay of the proenzyme to use some other method of activation. Streptokinase, a bacterial product, is available in a relatively purified state; has good stability and has high potency with respect to the degree of activation achieved, but it must be recognized that streptokinase may not have the same mode of action as the physiologic activators and that the degree of activity obtained may be in part dependent upon other factors in human plasma in addition to profibrinolysin. Mullertz and Lassen have recently concluded that streptokinase converts a prokinase of human plasma to an active kinase and that the plasmatic kinase is responsible for the transformation of profibrinolysin to fibrinolysin (23). The experimental findings of Mullertz and Lassen have been confirmed in this laboratory, but their interpretation is not the only one possible. As has been suggested by Kocholaty, Ellis, and Jensen, fibrinolysin may activate profibrinolysin in a similar manner to the activation of trypsinogen by trypsin (24). Notwithstanding the uncertainty concerning the mode of action of streptokinase, it offers the best presently available material for the activation of human profibrinolysin.

In addition to the question concerning the relationship of streptokinase activatable human profibrinolysin to physiologically activatable plasma profibrinolysin, the effect of possible contamination of the material assayed with antifibrinolysin must be considered. Antifibrinolysin titers have been shown to rise in a number of disease states (25–27), including cancer (26, 28, 29). Thus, if the isolation procedure for profibrinolysin does not free the product of this contaminant, it is logical to conclude that the fibrinolytic activity resulting from activation of the profibrinolysin is a function of at least two variables: the amount of profibrinolysin and the amount of antifibrinolysin in the native plasma. Since antifibrinolysin tends to increase in patients with malignant neoplasms, this contaminant could mask a real increase in profibrinolysin. Cliffton (27) has obtained data which he interprets as suggestive of a fall in both spontaneous and streptokinase activatable profibrinolytic activity in malignant neoplasms as compared to the other conditions tested. The inverse relationship between proteolytic and antiproteolytic titers could have resulted from the incomplete separation of antifibrinolysin, as Cliffton suggests. The method described in this report essentially removes the antifibrinolysin contaminant, thus eliminating this source of error. It is therefore believed that the results reported here more nearly approximate the true changes in profibrinolysin and that an actual statistical rise in profibrinolysin occurs in the patients with neoplasms and in pregnant women.

If proliferation of tissue were the primary fac-

tor in the alteration of the profibrinolysin-antifibrinolysin system in a patient with a neoplasm, other situations involving rapid development of new tissue might be expected to exhibit similar changes. Pregnancy appears to have certain aspects in common with neoplasia. In pregnancy there is a rapid proliferation of new tissue which is parasitic on the host and contributes nothing to the physiologic economy of the host organism. In both neoplasia and pregnancy the host organism supplies oxygen and nutrients to the rapidly proliferating tissues and products of metabolism of the parasitic tissues must be removed. The results reported here indicate that the changes in profibrinolysin, antifibrinolysin, and fibrinogen are in the same direction in neoplasia and pregnancy, but that the changes tend to be greater in pregnancy than in neoplasia. However, it cannot be assumed from these results that the only stimulus is cell proliferation, since many other factors, such as the oxygen available to the tissues of the host organism, stress, hormones, products of metabolism and of necrosing cells, etc., may be common to the two conditions. Nevertheless, the similarities in pregnancy and neoplasia and the similarities in response of the profibrinolysin-antifibrinolysin system suggest the use of pregnant women for a comparative study, especially since the stage of pregnancy can be readily determined and the patient can be made available for post partum control studies.

The lysis of fibrin clots, or hydrolysis of other proteins, in the presence of urine dialysates or concentrates at pH 7.2 to 7.4 has been reported from several laboratories. Williams (17) and Astrup and Sterndorff (18) have claimed that the breakdown of the fibrin clot is due almost entirely to the activation of a profibrinolysin contaminant in the fibrin substrate by a urine fibrinokinase. Recently in this laboratory evidence has been obtained that human urine and urine from other species have, in addition to fibrinokinase activity, a fibrinoprotease activity (19). This fibrinoprotease activity brings about the direct lysis of fibrin clots in the absence of profibrinolysin. It is emphasized, however, that the results reported here do not distinguish between kinase and protease, since bovine fibrinogen, prepared by the freeze-thawing technic, may occasionally be contaminated with traces of profibrinolysin. The assay reported here, therefore, only indicates total lytic potency of the urine samples. In some cases the lysis could have been largely due to activation of the profibrinolysin contaminant by urine kinase; in others, the lysis could have been the result of the direct lysis of bovine fibrin by urine fibrinoprotease, or both urine kinase and fibrinoprotease could contribute to the fibrinolytic end-point.

## SUMMARY

1. An assay for human plasma profibrinolysin is described in which the profibrinolysin is isolated through treatment with protamine sulfate, dilution and pH alteration. After activation with streptokinase the fibrinolytic activity is measured by the rate of lysis of a standard fibrin clot.

2. A method for measuring urine fibrinolytic activity is described. Salts are removed by dialysis and fibrinolytic activity is measured by the rate of lysis of a fibrin clot.

3. Plasma profibrinolysin, antifibrinolysin, and fibrinogen concentrations are increased in patients with neoplasms and in pregnant women when compared to the mean concentrations of these factors in normal individuals.

4. Plasma profibrinolysin, antifibrinolysin, and fibrinogen tend to show greater increases in pregnant women than in patients with neoplasia.

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# METHEMOGLOBIN FORMATION IN HUMAN BLOOD BY COBALT *IN VITRO* [1]

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(Submitted for publication October 15, 1953; accepted July 28, 1954)

Although the erythropoietic effect of cobalt has been amply demonstrated in experimental animals (1–3), in normal human beings (4, 5), and in certain types of anemia (4, 6–8), the mechanism of this increased blood formation has not been elucidated. No evidence has been adduced that administration of cobalt causes a decrease in oxygen capacity (9) or alters the oxygen dissociation curve (10, 11) in the blood either of experimental animals or of human beings. It has been suggested, therefore, that cobalt interferes with the cellular oxidative processes of the marrow, or of some other hypothetical regulatory center for erythropoiesis, thus producing a histotoxic anoxia (4), leading in turn to increased erythropoietic activity.

Some support for this hypothesis is offered by observations which show that cobalt induces the formation of reversible complexes of oxygen with histidine or cysteine (12, 13). Moreover, Barron and Barron (14) have shown that the addition of cobalt *in vitro* diminishes the oxygen consumption of suspensions of red cells derived from rabbits made polycythemic by the previous administration of cobalt. These authors and Davis (15) have also demonstrated that the development of cobalt-induced polycythemia is prevented by the simultaneous administration of ascorbic acid, a reducing agent known to be effective in directly reducing methemoglobin to hemoglobin (16). It seems possible, then, that cobalt may exert its histotoxic effect by blocking the enzymatic reducing systems of cells, thus interfering with the utilization of oxygen by the cells.

In normal intact erythrocytes *in vitro* methemoglobin is reconverted to hemoglobin spontaneously at the rate of 1.2 gm. per 100 cc. of blood per hour (17) by an enzymatic reducing system within the cells involving glucose and glycolysis (18–21). Consequently, it was considered appropriate to examine the action of cobalt in relation to the formation of methemoglobin in intact erythrocytes and to determine whether any methemoglobin thus formed was a result of direct oxidation of hemoglobin by cobalt or of an inactivation of the normal methemoglobin reducing mechanism.

## METHODS

Quantitative estimation of the methemoglobin in blood was performed by the method of Evelyn and Malloy (22). Examination of the pigments in blood was made with the Beckman Spectrophotometer, Model B. The pH of the blood was measured with a glass electrode on the Beckman pH Meter. Blood sugar was determined by the method of Somogyi (23).

## REAGENTS

*Cobaltous chloride.* "Baker's Analyzed" $CoCl_2 \cdot 6H_2O$; 4 gm. of the crystals were dissolved in 100 ml. of distilled water. The solution was autoclaved before use.

*p-Aminophenol.* Eastman Kodak Co.; 25 mg. were dissolved in 50 ml. of 0.85 gm. per cent NaCl. This 50 mg. per cent solution was freshly prepared immediately before use.

*Ascorbic acid.* Hoffmann-LaRoche, Inc. Vitamin C, Injectable; 1 ml. containing 250 mg. was diluted with 9 ml. of 0.85 gm. per cent NaCl to give a 2.5 gm. per cent solution. This dilution was freshly prepared immediately before use.

*Methylene blue.* National Aniline Pharmaceutical Laboratories; 100 mg. of this powder were dissolved in 100 ml. of 0.85 gm. per cent NaCl. This 0.1 gm. per cent solution was then diluted 1:9 with 0.85 gm. per cent NaCl to give an 0.01 gm. per cent solution.

*Glucose.* "Baker's Analyzed" Dextrose; 5 gm. were dissolved in 100 ml. of distilled water.

[1] This study was supported in part by grants from the American Cancer Society, Inc. and the Damon Runyan Memorial Fund for Cancer Research.

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*Hemoglobin crystals* (24). The red cells from 25 ml. of defibrinated blood from normal individuals were washed three times with 0.85 gm. per cent NaCl after the removal of the serum. The packed cells were then laked with twice their volume of distilled water, and 0.4 volume of toluene was added. After being kept in the refrigerator overnight, the mixture was centrifuged at 3,000 r.p.m. for 30 minutes. Both stroma and toluene were removed, and the supernatant hemoglobin solution was then placed in a cellophane sac and dialyzed against 1 liter of saturated ammonium sulfate solution for 24 hours at 4° C. The resultant precipitate had the microscopic appearance of hemoglobin crystals. The crystals thus prepared were made ready for use by dissolving in approximately 20 ml. of 0.85 gm. per cent NaCl.

## EXPERIMENTAL

### *Formation of Methemoglobin in Erythrocytes Incubated with Cobalt*

Blood was drawn from a normal subject and defibrinated under sterile conditions. The hematocrit was then adjusted to 50 per cent with the subject's serum, as it was in all subsequent experiments. Sixteen ml. of this blood were mixed with 0.8 ml. of 4 gm. per cent $CoCl_2$ (yielding a final concentration of cobaltous chloride of 0.2 per cent). Another 16 ml. were mixed with 0.8 ml. of 0.85 gm. per cent NaCl and one-half of each sample was incubated for 24 hours at 37° C. On the remainder of each sample the pH was determined and adjusted with $CO_2$ to approximately 7.0. The methemoglobin content of the unincubated samples and of the incubated samples after appropriate periods of incubation was measured and expressed as per cent of total hemoglobin.

The results of six experiments are shown in Table I and demonstrate that an appreciable amount of methemoglobin was formed in the cells incubated with cobalt. The identity of the pigment was confirmed by its light absorption at 635 millimicrons and the amount determined by the change in extinction upon the addition of neutralized sodium cyanide.

TABLE I

*Formation of methemoglobin upon incubation of cobaltous chloride with normal human defibrinated blood*

| Blood sample incubated with | Number of expts. | Hours at 37° C. | Methemoglobin % of total hemoglobin |
|---|---|---|---|
| Sodium chloride | 6 | 0 | 0 |
| | 6 | 24 | 0 to 0.85<br>Av. 0.53 |
| Cobaltous chloride | 6 | 0 | 0 |
| | 6 | 24 | 9.6 to 15.0<br>Av. 11.85 |

### *Action of Cobalt Contrasted with p-Aminophenol in the Formation of Methemoglobin in Erythrocytes*

The following experiments were designed to determine whether the formation of methemoglobin induced by cobaltous chloride in normal blood results from increased oxidation of hemoglobin or from inhibition of the normal reduction mechanism. The action of cobaltous chloride was, therefore, compared to that of p-aminophenol, an agent known to produce methemoglobin by accelerated oxidation of hemoglobin (25).

#### *The rate of formation of methemoglobin*

Ten ml. of normal defibrinated blood were mixed with 0.5 ml. of 4 gm. per cent $CoCl_2$ solution. A 5 ml. sample of the same blood was mixed with 5 ml. of freshly prepared 50 mg. per cent solution of p-aminophenol. An additional 5 ml. sample was mixed with 5 ml. of 0.85 gm. per cent NaCl. From each sample 13 aliquots of about 0.5 ml. were placed in separate 10 × 100 mm. test tubes, which were then placed in a water bath at 37° C. A methemoglobin determination was made from one of the 0.5 ml. aliquots of the three types of mixture every two hours for 24 hours.

The results of an experiment typical of the two performed are shown in Figure 1 and indicate striking differences in the rates of formation of methemoglobin. The formation of methemoglobin induced by p-aminophenol was rapid for the first few hours and then proceeded relatively slowly. No significant amount of methemoglobin was formed in the blood incubated with cobaltous chloride for the first 10 hours. Thereafter, methemoglobin formation gradually increased so that at the end of 24 hours 8 per cent of the hemoglobin had been oxidized to methemoglobin. In the sample incubated with NaCl, no methemoglobin was formed at the end of 24 hours.

#### *The effect of anoxia on methemoglobin formation*

It has been shown that in the presence of oxygen, p-aminophenol will directly oxidize the hemo-

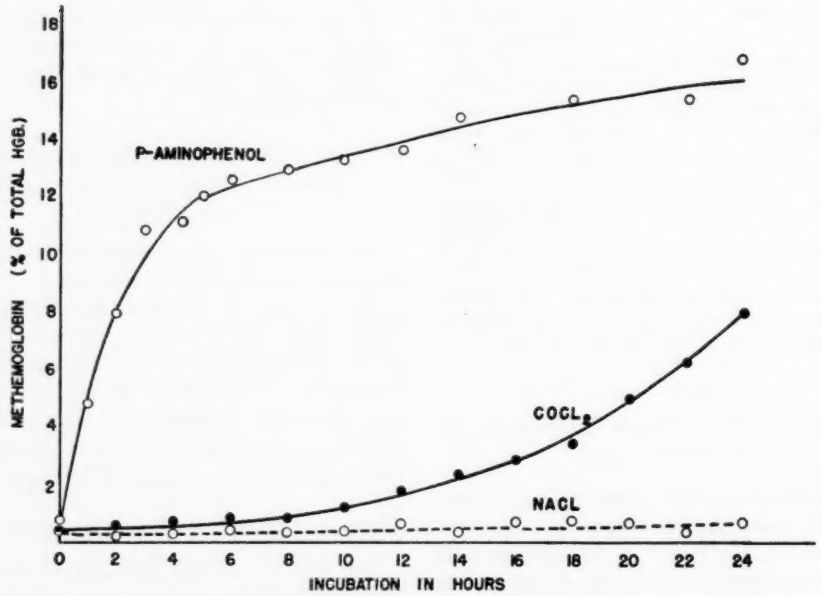


FIG. 1. RELATION BETWEEN METHEMOGLOBIN FORMATION AND DURATION OF INCUBATION OF HUMAN DEFIBRINATED BLOOD WITH P-AMINOPHENOL AND COBALTOUS CHLORIDE

globin of normal blood to methemoglobin, whereas in the absence of oxygen, little or no methemoglobin is formed (25). Methylene blue may either catalyze the oxidation of hemoglobin to methemoglobin or reduce the latter to hemoglobin (26). It was of interest, then, to study the effect of anoxia on the formation of methemoglobin by cobalt and especially in comparison with p-aminophenol and methylene blue.

Two 8 ml. samples of human defibrinated blood were mixed with 4 ml. of 0.85 gm. per cent NaCl. Two 8 ml. samples were mixed with 0.4 ml. of 0.01 gm. per cent methylene blue. Two 8 ml. samples were mixed with 4 ml. of 50 mg. per cent p-aminophenol. Finally, two 8 ml. samples were mixed with 0.4 ml. of 4 gm. per cent $CoCl_2$. The eight samples were then placed in tonometers; and one of each pair was equilibrated with a mixture of 90 per cent $O_2$ and 10 per cent $CO_2$, the other with 90 per cent $N_2$ and 10 per cent $CO_2$. All samples were then incubated for 24 hours at 37° C. At the end of the incubation period, methemoglobin determinations were done on each sample.

The results presented in Table II indicate that in the presence of oxygen, p-aminophenol and methylene blue readily formed methemoglobin; whereas, in the absence of oxygen, they formed relatively little methemoglobin. In contrast, cobalt permitted the formation of substantially more methemoglobin in the absence of oxygen than in the oxygenated sample. The control samples incubated with sodium chloride solution showed no significant amount of methemoglobin in either gas mixture.

TABLE II

*Effects of p-aminophenol and of cobaltous chloride upon methemoglobin formation in blood incubated in presence and in absence of oxygen*

| Blood sample incubated with | Number of expts. | Hours at 37° C. | Methemoglobin % of total hemoglobin | |
|---|---|---|---|---|
| | | | Gas mixture | |
| | | | 90% $O_2$ 10% $CO_2$ | 90% $N_2$ 10% $CO_2$ |
| Sodium chloride | 6 | 24 | 0 to 0.85 Av. 0.53 | 0 |
| Methylene blue | 2 | 24 | 8.2 and 13.6 Av. 10.9 | 2.8 and 6.7 Av. 4.7 |
| p-Aminophenol | 2 | 24 | 59.3 and 63.1 Av. 61.2 | 4.7 and 7.6 Av. 6.1 |
| Cobaltous chloride | 6 | 24 | 2.0 to 5.3 Av. 3.7 | 6.3 to 34.3 Av. 15.2 |

### *The effect of methylene blue on the formation of methemoglobin by cobalt and by p-aminophenol*

It has been demonstrated that methylene blue reduces preformed methemoglobin in intact erythrocytes by accelerating the normal reconversion mechanism (27, 28). Since the preceding experiments suggested that cobalt induces methemoglobin formation by inhibiting the normal reconversion mechanism, whereas p-aminophenol directly oxidizes hemoglobin to methemoglobin, the effects of cobalt and of p-aminophenol in the presence of appropriate amounts of methylene blue were contrasted.

To 10 ml. of normal human defibrinated blood were added 5 ml. of 50 mg. per cent p-aminophenol in 0.85 gm. per cent NaCl. To another 10 ml. sample, 0.5 ml. of 4 gm. per cent $CoCl_2$ was added. Each sample was then divided into two equal parts. To one part of each sample, 0.5 ml. of 0.85 gm. per cent NaCl containing 0.01 gm. per cent methylene blue was added; to the other, 0.5 ml. of 0.85 gm. per cent NaCl. The samples were then incubated at 37° C., and methemoglobin determinations were done on the samples containing p-aminophenol after 8 and after 24 hours and on those samples containing cobalt after 24 hours.

The results obtained at 24 hours presented in Table III indicate that the addition of methylene blue substantially prevented methemoglobin formation in the blood containing p-aminophenol. In contrast, methylene blue did not suppress methemoglobin formation in the blood containing cobalt.

TABLE III

*Effects of methylene blue on the formation of methemoglobin during incubation of normal blood with p-aminophenol and with cobaltous chloride*

| Blood sample incubated with | Number of expts. | Hours at 37° C. | Methemoglobin % of total hemoglobin |
|---|---|---|---|
| p-Aminophenol and Sodium chloride | 4 | 24 | 48.0 to 68.4 Av. 58.1 |
| p-Aminophenol and Methylene blue | 4 | 24 | 0.9 to 7.7 Av. 4.2 |
| Cobaltous chloride and Sodium chloride | 4 | 24 | 6.0 to 19.5 Av. 10.6 |
| Cobaltous chloride and Methylene blue | 4 | 24 | 8.1 to 18.2 Av. 13.8 |

TABLE IV

*Inhibitory action of ascorbic acid on the formation of methemoglobin during incubation of normal blood with cobaltous chloride or p-aminophenol*

| Blood sample incubated with | Number of expts. | Hours at 37° C. | Methemoglobin % of total hemoglobin |
|---|---|---|---|
| Sodium chloride | 4 | 24 | 0.4 to 0.7 Av. 0.6 |
| Cobaltous chloride and Sodium chloride | 6 | 24 | 7.1 to 15.0 Av. 11.5 |
| Cobaltous chloride and Ascorbic acid | 6 | 24 | 0 to 3.4 Av. 1.2 |
| p-Aminophenol and Sodium chloride | 5 | 24 | 29.7 to 71.3 Av. 44.6 |
| p-Aminophenol and Ascorbic acid | 5 | 24 | 1.1 to 8.1 Av. 2.8 |

### *The effect of ascorbic acid on the formation of methemoglobin by cobalt and by p-aminophenol*

In contrast to methylene blue, ascorbic acid has been shown to reduce methemoglobin directly rather than by way of the normal cellular enzymatic process (29). The following experiment was designed to determine whether ascorbic acid would prevent or inhibit methemoglobin formation induced by either cobalt or p-aminophenol.

To 10 ml. of normal human defibrinated blood 5 ml. of 50 mg. per cent p-aminophenol in 0.85 gm. per cent NaCl were added. To another 10 ml. of blood 0.5 ml. of 4 gm. per cent cobaltous chloride was added. Each sample was then divided into two parts. To one part was added 0.8 ml. of 2.5 gm. per cent solution of ascorbic acid; to the other was added 0.8 ml. of 0.85 gm. per cent NaCl. Another 5 ml. aliquot of blood was mixed with 0.8 ml. of 0.85 gm. per cent. NaCl to serve as a control. All samples were incubated at 37° C. Methemoglobin determinations were done on all samples at the end of 8 hours and of 24 hours.

The results presented in Table IV indicate that the addition of ascorbic acid markedly inhibits the formation of methemoglobin by either cobalt or p-aminophenol.

### *Formation of Methemoglobin in a Solution of Hemoglobin Crystals Incubated with Cobalt*

If the transformation of hemoglobin to methemoglobin by the action of cobalt is due entirely to

the inhibition of the cellular reconversion mechanisms, the exposure of crystalline hemoglobin to cobalt, in the absence of the requisite enzymes and their substrates, should produce no more methemoglobin than exposure to any inert substance. The following experiment was done to test this assumption.

Hemoglobin crystals prepared from 20 ml. of normal defibrinated blood as described above were dissolved in 20 ml. of sterile distilled water and the solution divided into two parts. To one part was added 0.5 ml. of 0.85 gm. per cent NaCl and to the other 0.5 ml. of 4 gm. per cent $CoCl_2$. Each part was then further divided into 0.5 ml. aliquots, which were placed in 10 × 100 mm. test tubes and incubated at 37° C. Methemoglobin determinations were done at appropriate intervals during 24 hours.

The results of an experiment typical of four are plotted in Figure 2 and show that the addition of cobaltous chloride to the solution of hemoglobin gave no significantly greater yield of methemoglobin than did the addition of sodium chloride.

## DISCUSSION

In normal erythrocytes, both in the circulation and *in vitro*, hemoglobin is constantly being oxidized to methemoglobin presumably by the process of auto-oxidation (26). The methemoglobin thus formed is constantly being reconverted to hemoglobin, chiefly by an enzymatic process which functions so efficiently that both *in vivo* and *in vitro* (26) only small amounts of methemoglobin can be found as long as the necessary glycolytic enzymes and their substrates are present. In addition to this process involving glycolysis, methemoglobin may also be reduced directly by the action of several agents, such as ascorbic acid, cysteine, glutathione, glyceraldehyde, and BAL (dimercaptopropanol) (26, 30).

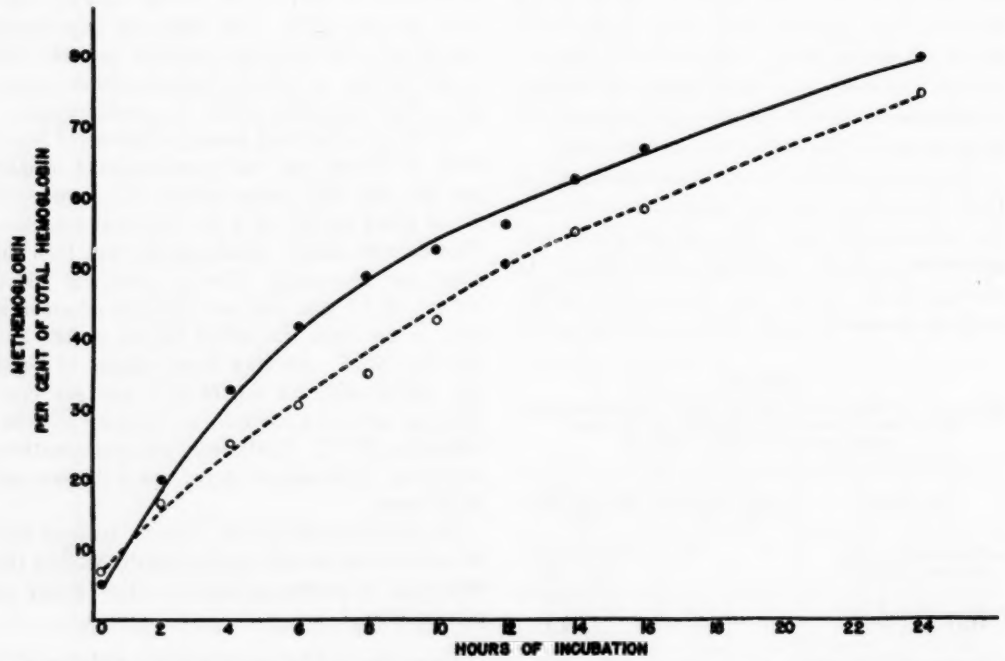


FIG. 2. FAILURE OF COBALT TO AUGMENT THE FORMATION OF METHEMOGLOBIN IN A SOLUTION OF HEMOGLOBIN CRYSTALS INCUBATED IN ROOM AIR AT 37° C.

○—○ Hemoglobin crystals in water and NaCl.
●—● Hemoglobin crystals in water and $CoCl_2$.

Under abnormal circumstances methemoglobin may accumulate in erythrocytes due either to an acceleration of the oxidative process beyond the capacity of the normal reduction mechanisms or to a failure of the reduction mechanisms in the presence of the normal oxidative process. It would appear from the experiments cited above that the formation of methemoglobin in blood exposed to cobaltous chloride may best be explained by the latter mechanism. Comparison of the methemoglobin formation by p-aminophenol with that by cobalt, with respect to both rate of formation and effect of anoxia (Table I and II), strongly implies that cobalt does not produce methemoglobin by the same mechanism as does p-aminophenol, a substance known to oxidize hemoglobin directly. Methylene blue both *in vitro* and *in vivo* may, in appropriate concentrations, either accelerate the formation of methemoglobin or catalyze its reduction (26). The former action presumably explains the moderately enhanced formation of methemoglobin by methylene blue alone in oxygen illustrated in Table II. In the first portion of Table III the opposite effect of methylene blue in inhibiting the oxidizing action of p-aminophenol is clearly shown.

Since the reduction of methemoglobin by methylene blue depends on an intact enzymatic reduction system (27, 28), the failure of methylene blue to reduce the methemoglobin induced by cobalt illustrated in the second portion of Table III implies an interference by cobalt with this reduction system. That the methemoglobin so formed may be reduced in the presence of cobalt without participation of the enzymatic system is demonstrated by the action of ascorbic acid (Table IV), an agent known to reduce methemoglobin directly. Finally, when hemoglobin is purified and the enzymatic reduction mechanism is no longer present to be inhibited by cobalt, no appreciably greater amounts of methemoglobin are induced by incubation with cobalt than with an inert substance, such as NaCl (Figure 2).

The implication that cobalt interferes with this normal enzymatic reduction system *in vitro* may be of importance in relation to the action of cobalt in stimulating erythropoiesis *in vivo*. If erythropoietic activity depends on the relative oxygen saturation of the marrow cells, or of some other hypothetical regulatory center, erythropoietic activity will be stimulated by any agent which interferes either with the delivery of oxygen to or with the uptake or utilization of oxygen by the regulatory cells (31). An agent which blocks intracellular enzymatic reduction systems and so inhibits the utilization of oxygen might thus cause histotoxic anoxia of the regulatory cells and so lead to increased erythropoiesis, even though the supply of oxygen brought to these cells was within normal limits. It would appear from the observations reported above that cobalt does in fact inhibit the enzymatic reduction system concerned with the reconversion of methemoglobin to hemoglobin. The failure to find increased levels of methemoglobin in the blood of patients and experimental animals treated with cobalt makes doubtful a relationship between the inhibition of this particular enzyme system of the adult red cell and the development of cobalt polycythemia. Nevertheless, a similar inhibitory effect by cobalt in other and perhaps more sensitive intracellular enzyme systems of other cells might well eventuate there in histotoxic anoxia and hence in erythropoietic stimulation.

## SUMMARY

Normal human blood was incubated at 37° C. for 24 hours in the presence of 0.2 per cent cobaltous chloride. Formation of methemoglobin was observed.

Study of the mechanism of methemoglobin formation under these circumstances suggests that cobalt inhibits the normal intracellular reduction system which maintains hemoglobin in the ferrous state.

Others have shown that administration of cobalt to normal animals or man results in polycythemia without evidence of methemoglobin formation or decrease in oxygen capacity of the peripheral blood. Consequently, the physiological relevance of the present observations to such enhanced erythropoiesis is not direct. However, they are at least consistent with the hypothesis that cobalt, by its interference with enzymatic reduction systems, induces relative anoxia of tissues, including those controlling erythropoiesis, and thus may stimulate production of erythrocytes.

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# THE CHOICE OF SKIN AREA FOR TOXIN ASSAY

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(Submitted for publication December 16, 1953; accepted June 25, 1954)

A number of toxins have been assayed by measurement of lesions produced in small animals after intradermal injection of the toxin. The skin areas injected have been chosen largely for ease of injection, but incomplete studies have been made of which areas are most sensitive and which give the least variable responses. In the present paper the relative sensitivity and variability of different skin areas have been determined when intradermal hemorrhages are produced in the guinea pig by injection of Witte's peptone.

## METHODS

Seven young guinea pigs (200 to 250 gm.) were depilated by shaving or with depilatory powder shortly before use. The skin of the abdomen, back, and flanks was marked off in squares with India Ink. Witte's peptone, dissolved in saline, was then injected in three concentrations in 1/16 cc. volume according to Figure 1. This pattern represents an endless series of Latin Squares of the same design. Two hours after injection the diameters of the resulting intradermal hemorrhages were measured immediately after exsanguination. The lesions were usually sharply demarcated. In four of these animals trypan blue was injected intravenously before skin injections were made, and the diameter of the discolored area also measured.

A classical Latin Square analysis was carried out for each animal according to Fisher (1). Rows I, II, and III (Figure 1) produce three Latin Squares which overlap with the three squares produced by rows II, III, and IV; and each set of three squares was treated with a combined analysis of the three squares. The two sets of data share two-thirds of the raw data in common. It is not possible to combine these two sets of data into a single analysis inasmuch as the Latin Square design is a three by three design and cannot accommodate a fourth row. The two sets of data are, therefore, not independent of each other and it is to be expected that the results would agree closely. Neither was it feasible to plan larger Latin Squares, as only incomplete squares would have been left for a revised analysis after eliminating the variable areas. In view of the homogeneity of the results of these individual animal analyses, a combined analysis for the seven animals was carried out (2). In these analyses the square root of the diameter, expressed in millimeters, was used as explained elsewhere (3).

## RESULTS

The results are tabulated in Table I, which is laid out in the same way as Figure 1, and each square corresponds to a single square in Figure 1. The sum of the responses of all seven guinea pigs in each location is indicated in the individual values for each square.

The analysis in Table II indicates a sizeable error due to a column-square interaction (*i.e.*, individual column differences vary from square to square.) By inspection of the raw data in Table I, it is clear that the two columns representing the ventral area are the source of the variability. However, this variability does not appear in the statistical analysis as a "between columns" effect inasmuch as the "between columns" effect is made up of the sum of Columns A, D, and G compared with Columns B, E, H, and Columns C, F, and I; thus, the significantly variable areas in the abdominal region, namely Columns A and I are submerged in other relatively non-variable values. In the same way the "between row" variation will not disclose any unusual variation because values from Column A and I are equally distributed in each of the rows; however, the discrepancy due to the abdominal variation is evident in that portion of the analysis in which the effect of individual column totals is considered, and that is in the "column square" interaction of this analysis. Most lesions in the ventral area are much smaller than those on the back, and there is considerable variability. When the analysis is carried out (Table III) omitting the ventral skin and the single column in the middle of the back (the latter for convenience only) the resulting values show no significant variation due to position.

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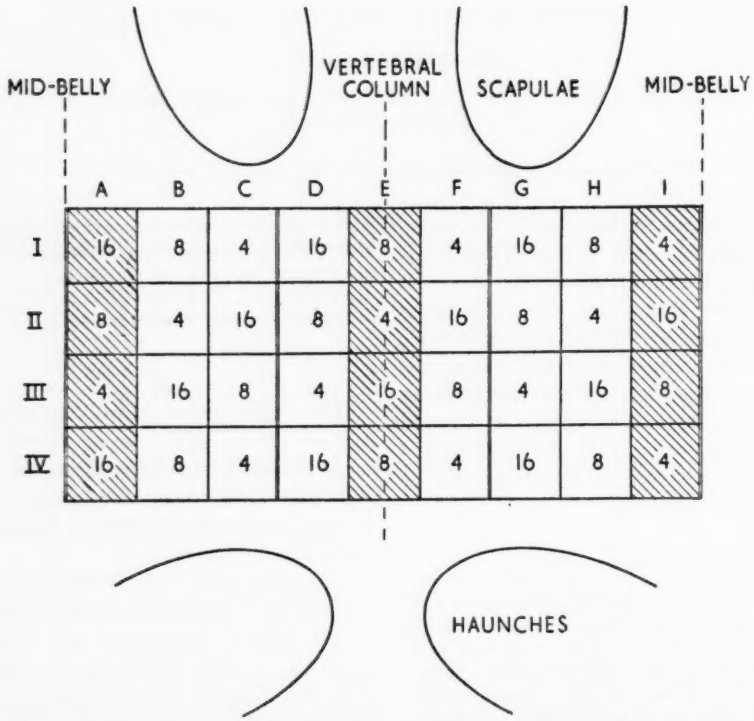
MID-BELLY
VERTEBRAL COLUMN
SCAPULAE
MID-BELLY
A B C D E F G H I
I 16 8 4 16 8 4 16 8 4
II 8 4 16 8 4 16 8 4 16
III 4 16 8 4 16 8 4 16 8
IV 16 8 4 16 8 4 16 8 4
HAUNCHES

FIG. 1. SCHEMATIC REPRESENTATION OF THE SKIN OF A GUINEA PIG INCLUDING THE AREAS USED FOR INJECTION

The numerals refer to the concentration of peptone injected in mg. per ml. of peptone nitrogen. The groups of 12 squares set off by double lines represent three sets of two overlapping Latin Squares used in the analysis (*e.g.*, A, B, C—I, II, III, and A, B, C—II, III, IV). The cross-hatched squares represent the areas discarded in final use of the test.

TABLE I

*The total diameters of skin hemorrhages produced by peptone in seven guinea pigs injected intradermally according to Figure 1* *

| | A | B | C | D | E | F | G | H | I | |
|---|---|---|---|---|---|---|---|---|---|---|
| Totals for columns I, II, III | 29<br>*15.03* | 60<br>*25.71* | 75<br>*35.71* | 49<br>*23.48* | 57<br>*25.64* | 62<br>*30.70* | 50<br>*25.46* | 66<br>*27.95* | 36<br>*16.51* | 484<br>*226.19* |
| I | 25<br>*11.62* | 13<br>*7.69* | 7<br>*5.83* | 37<br>*15.92* | 17<br>*8.18* | 8<br>*5.66* | 36<br>*15.75* | 13<br>*7.15* | 13<br>*5.06* | 169<br>*82.86* |
| II | 2<br>*2.00* | 0<br>*0* | 48<br>*18.02* | 12<br>*11.63* | 2<br>*7.56* | 40<br>*16.70* | 9<br>*5.97* | 2<br>*2.00* | 21<br>*10.03* | 136<br>*63.94* |
| III | 2<br>*1.41* | 47<br>*18.02* | 20<br>*11.63* | 0<br>*0* | 38<br>*16.04* | 14<br>*8.34* | 5<br>*3.73* | 51<br>*18.81* | 2<br>*1.41* | 179<br>*79.40* |
| IV | 25<br>*11.96* | 9<br>*4.86* | 4<br>*3.41* | 44<br>*17.37* | 11<br>*5.70* | 6<br>*4.15* | 43<br>*17.29* | 21<br>*11.11* | 5<br>*3.15* | 168<br>*79.01* |
| Totals for columns II, III, IV | 29<br>*15.38* | 56<br>*22.88* | 72<br>*33.29* | 56<br>*24.93* | 51<br>*23.16* | 60<br>*29.19* | 57<br>*26.99* | 74<br>*31.92* | 28<br>*14.59* | 483<br>*222.34* |

* The upper values are arithmetic totals; the lower values in italics are totals of the square roots of the raw values.

TABLE II

*The analysis of the Latin Squares when all columns are included* †

| | Rows | Degrees of freedom | Sum of squares | Mean square | Ratio |
|---|---|---|---|---|---|
| Rows | I, II, III | 2 | 22.56 | 11.28 | 2.47 |
| | II, III, IV | | 17.27 | 8.64 | 1.97 |
| Columns | I, II, III | 2 | 22.48 | 11.24 | 2.46 |
| | II, III, IV | | 7.77 | 3.88 | 1.13 |
| Treatments | I, II, III | 2 | 787.85 | 393.92 | 86.34*** |
| | II, III, IV | | 911.09 | 455.54 | 103.69*** |
| Squares | I, II, III | 2 | 5.64 | 2.82 | 1.62 |
| | II, III, IV | | 1.88 | .94 | 4.66 |
| Row-square interaction | I, II, III | 4 | 18.93 | 4.73 | 1.00 |
| | II, III, IV | | 40.76 | 10.19 | 4.16 |
| Column-square interaction | I, II, III | 4 | 82.08 | 20.52 | 4.50* |
| | II, III, IV | | 105.78 | 26.44 | 10.79** |
| Treatment-square interaction | I, II, III | 4 | 16.54 | 4.14 | 1.14 |
| | II, III, IV | | 6.03 | 1.51 | 1.63 |
| Error | I, II, III | 6 | 28.40 | 4.73 | |
| | II, III, IV | | 14.71 | 2.45 | |
| Total | I, II, III | 26 | 984.49 | | |
| | II, III, IV | | 1,105.30 | | |
| Combined error | I, II, III | 14 | 63.87 | 4.56 | |
| | II, III, IV | | 61.51 | 4.39 | |

† Rows I, II, III were divided into three Latin Squares ABC, DEF, and GHI. Levels of significance are indicated in the column labelled "Ratio."

* Indicates a p value of less than 0.05.

** Indicates a value of less than 0.01.

*** Indicates a value of less than 0.001.

The underlined values are inverse ratios.

The accuracy obtained in assay procedures is conveniently expressed as the standard error of potency ($S_M$) (4). An analysis of variance of the results from the seven animals indicates that in a single animal the $S_M$ for an assay using thirty-six injections in all the areas indicated in Figure 1 would be approximately .25, and after conversion to arithmetic units would be .56. The $S_M$ of the eight ventral areas above would be .67 and after conversion 1.79. The $S_M$ of the 28 back areas above would be .22 and after conversion .48, so that with fewer injections (omitting the ventral area) greater accuracy is achieved.

The area of the blue discoloration in the four animals treated with dye was also smaller in the ventral area, but not significantly so.

## DISCUSSION

The assay of dermatonecrotic and erythrogenic bacterial toxins is customarily carried out in the back, flank, or ventral skin of rabbits or guinea pigs; the choice apparently dictated by ease of injection or tradition (5, 6). Wadley (7) has reported that different areas of cow hide have different sensitivity to tuberculin. Differing sensitivities to histamine and allergens in various human skin areas have been observed by Swain and Becker (8) and they have reviewed previous similar studies in humans. The toxins arising from proteolytic processes have also been tested in the skin of these animals. Menkin (9) and others (10–16) injected preparations of leukotaxine, enzymes, venoms, or other irritants into the ventral skin or back (17) of rabbits and measured grossly the size of the blue spots due to the escape of intravenously injected dye into these areas.

Miles and Miles (18) used a Latin Square design on the dorsal skin of guinea pigs to show lack of important variability in that area in responses to histamine, histamine-liberator, and leukotaxine with the dye technique. They reported larger lesions in the thinner ventral skin, which is in con-

TABLE III

*The analysis of the Latin Squares when only columns BCD and FGH are included, thus eliminating the belly and the mid-line of the back* †

| | Rows | Degrees of freedom | Sum of squares | Mean square | Ratio |
|---|---|---|---|---|---|
| Rows | I, II, III | 2 | 9.07 | 4.54 | 1.03 |
| | II, III, IV | | 9.19 | 4.59 | 1.17 |
| Columns | I, II, III | 2 | 7.89 | 3.94 | 1.12 |
| | II, III, IV | | 5.67 | 2.84 | 1.39 |
| Treatments | I, II, III | 2 | 635.72 | 317.86 | 71.92*** |
| | II, III, IV | | 735.04 | 367.52 | 93.35*** |
| Squares | I, II, III | 1 | .04 | .04 | 126.27 |
| | II, III, IV | | 2.72 | 2.72 | 1.45 |
| Row-square | I, II, III | 2 | .57 | .28 | 7.49 |
| interaction | II, III, IV | | 5.68 | 2.84 | 1.10 |
| Column-square | I, II, III | 2 | 24.96 | 12.48 | 5.86 |
| interaction | II, III, IV | | 18.67 | 9.33 | 3.61 |
| Treatment-square | I, II, III | 2 | 10.15 | 5.08 | 2.38 |
| interaction | II, III, IV | | 4.69 | 2.34 | 1.10 |
| Error | I, II, III | 4 | 8.51 | 2.13 | |
| | II, III, IV | | 10.34 | 2.58 | |
| Total | I, II, III | 17 | 696.91 | | |
| | II, III, IV | | 791.99 | | |
| Combined error | I, II, III | 10 | 44.20 | 4.42 | |
| | II, III, IV | | 39.37 | 3.94 | |

† There is now no significant column effect in the Column-square interaction.
*** Indicates a value of less than 0.001.

trast to the findings of the present experiments. Moore and Tobin (19) mapped skin areas in which radioactive dyes preferentially accumulated as a result of inflammation, but no systematic analysis of all trunk areas has previously been reported.

The Latin Square analysis is especially well suited for determining spatial variability. Originally designed for agricultural field experiments (1), it offers a simple and expedient means of determining which skin areas are least variable and most sensitive for skin assay.

An analysis similar to this might be performed for any given toxin, though it is not unlikely that the back of guinea pigs may prove the least variable area in its responses to a wide range of proteolytic products and bacterial toxins.

## SUMMARY

1. Witte's peptone produces hemorrhagic lesions in the skin of guinea pigs when injected intradermally.

2. By means of a Latin Square analysis of skin area, it has been found that the back shows no significant variability in its response, whereas the ventral skin is less sensitive than the back and, if included, contributes considerable variability and lack of precision.

3. Greater accuracy may be obtained in the skin assay of toxins if a Latin Square analysis be performed for the particular animal and toxin concerned.

## ACKNOWLEDGMENTS

Th author is indebted to Dr. William Reynolds of the Harvard School of Public Health for help with the statistical analysis.

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# THE PREPARATION AND ASSAY OF TOXIC PROTEOLYTIC PRODUCTS PRODUCING SKIN HEMORRHAGE [1]

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(Submitted for publication December 16, 1953; accepted July 7, 1954)

In attempting to purify toxic factors in peptones, it was found that adequate methods of bioassay were lacking. In particular, methods for assaying the potency of biological preparations which increase capillary permeability or produce hemorrhage in the skin have been crude and inaccurate. Commonly, a guinea pig or rabbit is prepared by the intravenous injection of a protein-binding (1–3) indicator; *e.g.*, sulfonated dyes such as trypan blue (4–9), fluorescent dyes (10), radioactive dyes (8), and certain metals (11, 12). The toxic material is then injected intradermally. An increase in capillary permeabiltiy is indicated by an accumulation of the indicator-protein complex at injection sites.

The intensity of the discoloration after intravenous dye has been estimated roughly using a + to ++++ scale (4–6, 9), or by measurements of diameter of the discolored area (7, 13, 14). However, the subjective evaluation of such diffuse and irregularly discolored areas is unsatisfactory for quantitative assay.

A method is presented in this paper for quantitative assay of toxins which produce local hemorrhage after intradermal injection. The amount of hemorrhage has been measured spectrophotometrically. Dosage-response curves have been prepared and ordinates obtained which give a linear relationship and equal variance at all dosage levels. Using these ordinates a five point slope-ratio assay is described which has an error ("standard error of potency") of approximately 11 per cent. This assay method has been used to study the appearance of toxic products during the tryptic digestion of fibrin.

## MATERIALS AND METHODS

*Materials.* The "standard" against which all unknowns were assayed was the non-ultrafiltrable (7½ per cent collodion membrane) portion of a six-hour tryptic digest of fibrin (see under Tryptic Digestion). In the standard preparation about half the total material was ultrafiltrable. Crystalline trypsin, polymyxin [2] (a large polypeptide), and kallikrein [3] (an insulin-free pancreatic extract) produce similar hemorrhages (15).

*Injection.* Only the backs of albino guinea pigs were used because preliminary work (16) established that the use of the abdominal skin introduces much greater variability of results. Animals weighing about 300 grams were depilated with barium sulfide paste shortly before injection. Test material was injected intracutaneously in a volume of 1/16 ml. from ¼ ml. syringes with No. 27 flat bevel needles. The order and site of injection were distributed at random (17), and all materials were assigned a code number. The code was not broken until measurements had been recorded.

*Indicator system.* The fixation of intravenously injected dye was first tried to indicate the extent of reaction to intradermally injected material. The reactions were not well circumscribed, and measurements of diameter were difficult and variable. The fixation of intravenously injected metal was also demonstrated by quantitative metal analyses in individual skin areas, but the variability was even greater than with the dye method. It was also observed that reddish-purplish hemorrhagic lesions appeared (Figure 1) within ten minutes of injecting these fractions intradermally, which reached maximal size after fifteen to thirty minutes. The amount of this hemorrhage in each site proved to be an excellent indicator of the amount of toxin injected.

*Measurements.* The diameters of these hemorrhages were measured on the intact animal after exsanguination following a blow on the head. This preserved normal tension of the skin in a better fashion than was the case when the skin was removed. In order to have an objective check on these measurements, a colorimetric method was used. The skin was removed and the individual areas placed in ten ml. of 1N NaOH at 37° for

[1] This work was supported in part by grants from the United States Public Health Service, the Milton Fund of Harvard University, and the Mallinckrodt Chemical Company.

[2] Polymyxin was supplied by Burroughs-Wellcome (USA).

[3] Kallikrein was supplied by Sterling-Winthrop Research Institute.

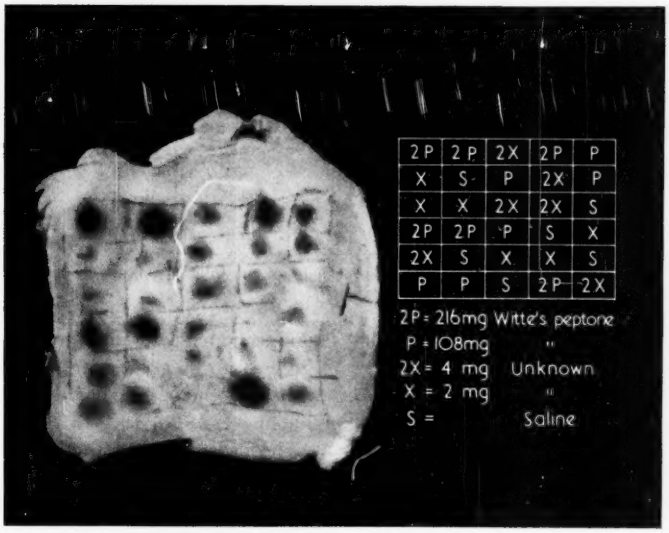


FIG. 1. TYPICAL ARRANGEMENT FOR ASSAY OF NON-FILTRABLE MATERIAL OF UNKNOWN POTENCY OBTAINED FROM TRYPTIC DIGESTION

The injections are distributed at random in space and time.

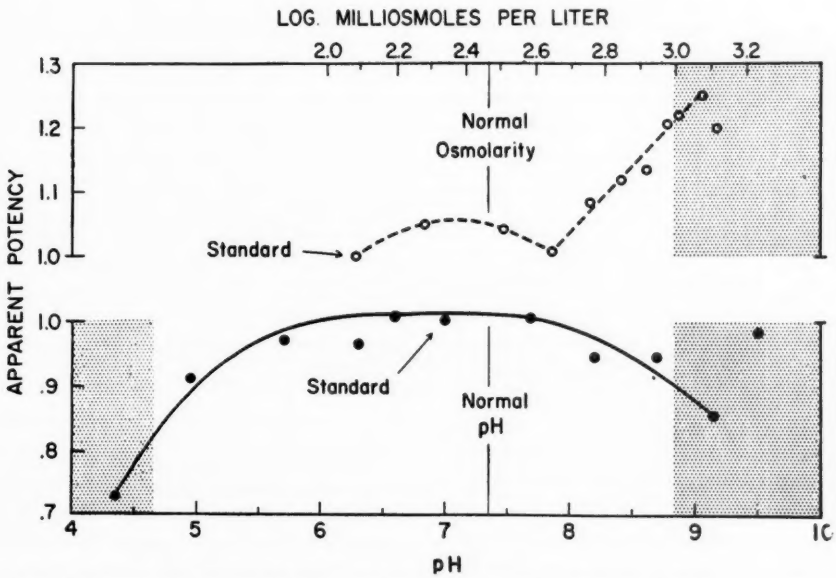


FIG. 2. EFFECT OF pH AND OSMOLAR CONCENTRATION ON THE APPARENT POTENCY OF NON-FILTRABLE PRODUCTS OF A TRYPTIC DIGEST OF FIBRIN

In the pH experiment, the osmolar concentration was constant, and the pH of the standard was adjusted to pH 7. In the osmolar experiment concentration of the standard was 125 mOs per L. The shaded areas indicate the extremes where a blanched (presumably avascular) lesion appeared. The standard error of the potency determinations varied between .017 and .055.

TABLE I

*Constitution of unknowns in 45 animal experiments* *

| Unknowns (4 injections each) | Experimental groups (5 animals each) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII | IX |
| A | S | S | S | S | S | S | S | S | S |
| B | 2 | 4 | 8 | 16 | 2 | 2 | 2 | 4 | 8 |
| C | 2 | 4 | 8 | 16 | 4 | 2 | 2 | 4 | 8 |
| D | 2 | 4 | 8 | 16 | 8 | 16 | 4 | 8 | 16 |
| E | 2 | 4 | 8 | 16 | 16 | 16 | 4 | 8 | 16 |

* Digits refer to concentration of "standard" peptone in mg. per ml. in each unknown. S = normal saline. Total of 900 injections, 20 to animal.

48 hours. The resulting digest was then filtered with the aid of diatomaceous earth ("super cell") and the alkaline hematin in the filtrate measured in a Coleman Junior spectrophotometer at 4000 and 4200 Å.

*Osmolar concentration and pH.* To determine the effect of variations in osmolar concentration (18), a single concentration of the standard was made up with varying amounts of sodium chloride at pH 7.0. The osmolar concentration was estimated by freezing point depression.[4] Thirty-six injections were then made in each of eight guinea pigs. In Figure 2, it is evident that between 125 and 570 milliosmoles per liter there is a minimal effect on apparent potency.

Similarly, to evaluate the effect of variations in pH, a single concentration of the standard was made up at varying pH but with a constant osmolar concentration (275–280 milliosmoles per Liter). Figure 2 indicates minimal effects of pH between pH 6.0 and 8.0. Solutions were maintained within these limits in later experiments.

## ASSAY TECHNIQUE

### *Dosage-response curve*

The amount of hemorrhage produced with different amounts of the standard preparation was studied so that ordinates could be found which would result in equal variance at all dosage levels, as well as providing a straight line relationship in some portion of the curve. Various concentrations of the standard and of saline controls were injected in 45 guinea pigs. Each pig received twenty assorted injections according to nine different designs which combined the five different dosage levels in various ways (Table I). These different designs were used in order to rule out any possible influence of one injection area on adjacent areas, or of the response to one dosage level to influence the response to another dosage level. The analysis of these results did not indicate any significant tendency for the responses to one dosage level to be greater or smaller when combined with any other particular dosage levels in a given animal.

The dosage response curve and its error were analyzed according to Bliss (19). In Figure 3, at the left, the dose is plotted against both the diameter of the hemorrhagic lesions and against the spectrophotometric readings. Although a straight line is obtained with the diameters of the hemorrhage, the variance with both methods of measurement is very much greater at higher dosage levels. The variance of the direct measurement of diameters of the hemorrhage is as much equalized at all dosage levels with a square root transformation as with any other root or logarithmic transformation. A square root transformation of the dosage is needed to achieve a straight line (Figure 3, at right). The variance of the spectrophotometric readings was satisfactorily adjusted with fourth root transformation, and a linear function obtained by raising the dosage to the 1.3 power. These manipulations used to obtain a straight line and equal variance at each dosage level greatly facilitate the assay calculations described below.

### *Assay of potency of unknown preparations*

With the information obtained from these studies it is possible to estimate the average error that will be obtained in assays of potency (19). The standard error of the dosage response line is in response units. This standard error must be converted into dosage units, because in assay procedures it is important to know how much error there is in estimates of the dose. The standard error (response units) of the line converted to dosage units is given the symbol $\lambda$. This term may then be converted

[4] These determinations were provided through the courtesy of Dr. Alexander Leaf.

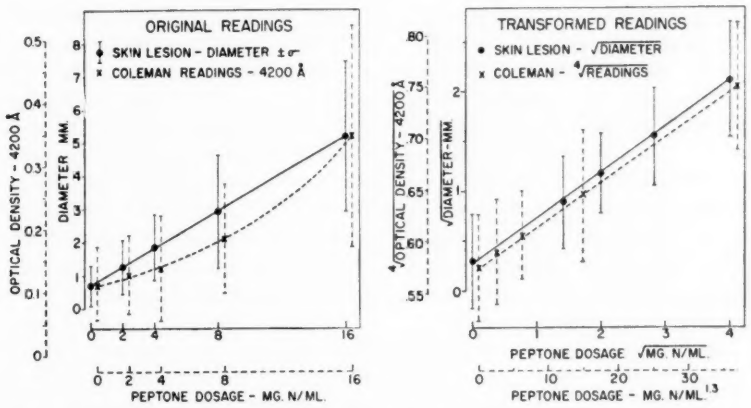


FIG. 3. DOSAGE-RESPONSE CURVES RELATING SKIN HEMORRHAGE TO PEPTONE DOSAGE

On the left are the original readings for both direct measurement of diameter, and for spectrophotometric readings of alkaline hematin present in alkaline digests of individual skin areas. The standard deviation for individual reactions at each dosage level is indicated by vertical lines. On the right, the same material has been transformed by response ordinates which roughly equalize the variance at all dosage levels, and by dosage ordinates which produce a linear function.

into the standard error of potency with the formula $S.E._{Potency} = 2\lambda\sqrt{N}$. When thirty injections are used in a given animal, the standard error of potency can then be calculated according to this formula as indicated in Table II. After the standard error has been expressed in terms of the transformed ordinates it must be converted to arithmetic units to indicate the actual error. Thus, in arithmetic terms the standard error of potency for direct readings is about 20 per cent, and for the spectrophotometric method about 12 per cent with readings either at 4000 Å or 4200 Å.

This expected error of assay determinations is useful if numerous assays are to be done, for one can spot repeated excessive error which may indicate carelessness or a change in technique. It can also be compared with that obtained for other assay procedures for comparing the relative efficiency of each. In Table II, the standard error of potency for a method using the measurement of diameter of hemorrhage is seen to be about the same as those obtained in measurements of diameter of tuberculin reactions (13) or of dye discolored spots (7). The objective spectrophoto-

TABLE II

*Standard error of potency assuming a potency of 1.00 for different methods of measurement*

| | λ | S.E. of potency $2\lambda/\sqrt{30}$ | 1 ± S.E. | Transformation to yield arithmetic values | 1 ± S.E. | % Error |
|---|---|---|---|---|---|---|
| Direct reading | .2664 | .0973 | 1.0973<br>.9027 | $(1 \pm S.E.)^2$ | +1.20<br>− .81 | +20<br>−19 |
| Spectrophotometer<br>4000 Å | .3850 | .1406 | 1.1406<br>.8594 | $\sqrt[1.3]{1 \pm S.E.}$ | +1.11<br>− .87 | +11<br>−13 |
| 4200 Å | .3830 | .1398 | 1.1398<br>.8602 | $\sqrt[1.3]{1 \pm S.E.}$ | +1.11<br>− .88 | +11<br>−12 |
| Dye Method:<br>Data of Miles and Miles (7) | .273 | .0968 | 1.0968<br>.9032 | antilog 1 ± S.E. | +1.25<br>− .80 | +25<br>−20 |
| Tuberculin:<br>Data of Wadley (13) | .264 | .0935 | 1.0935<br>.9065 | antilog 1 ± S.E. | +1.24<br>− .81 | +24<br>−19 |

metric method yields an appreciably smaller error.

A five-point slope-ratio assay (20) of a particular unknown may conveniently be carried out in a single guinea pig with two dilutions of unknown, two dilutions of standard, as well as saline controls (Figure 1). An advantage of obtaining a linear function with power or root ordinates, if the line goes through zero dose, is that a slope-ratio assay may be used which is more efficient than a parallel slope assay with logarithmic ordinates, because zero dose is common to both standard and unknown lines with slope ratio assays.

*Discussion*

The hemorrhage which is produced in skin by intradermal injection of proteolytic products is probably closely related to the escape of protein-bound dyes into these areas. The dye escape has been interpreted as indicating increased capillary permeability ever since the observation of Hoffman and Recklinghausen in 1867 (21) that cinnebar localized in an area of induced keratitis in the frog (10, 22–24). Erythrocytes appear in the extravascular spaces after intradermal injection of higher concentrations of many of the same toxins as promote escape of dye. The erythrocytes probably are squeezed out between endothelial cells, but no frank rupture of the endothelium has been observed in pathological sections. The induced disorder of the endothelium may then be termed increased capillary permeability or fragility, depending on whether permeability may include intercellular escape or is confined to escape through endothelial cells.

The mechanism for this erythrocyte escape is unknown, but it is known to result from the injection of hypertonic solutions locally (25, 26), from muscular activity alone (27), from marked tissue damage (25, 28), from irritant substances injected intra-arterially (29), from anaphylactic reactions (30–32), in the Schwartzman phenomenon (33), and in scurvy, hemophilia, thrombocytopenic purpura, and certain intoxications (34, 35). Proteolytic products may produce hemorrhage by activating tissue enzymes, in view of the observation that trypsin alone produces similar hemorrhages. However, it is possible that trypsin acts by releasing from tissue proteolytic products which are in themselves toxic.

The advantages of using the erythrocyte as the indicator in assay procedures are several. No preparative injections are needed as with the dye technique. Artificial indicators must be injected before intradermal test injections are made because increases in capillary permeability under experimental conditions are usually short-lived (7, 36–38); furthermore, the serum level of the indicator is not constant, so that whether the dye is injected before or after the test substance, inequalities are invariably introduced.

A variety of ordinates have been used to obtain linear dosage response curves of skin reactions to various irritants (7, 13, 14, 39–44). This diversity of results compared with the present findings suggests that any of several ordinates may produce a straight line over some portion of the regression curve.

TOXIN PREPARATION

*Tryptic digestion*

Five hundred grams of crude commercial fibrin was washed by repeated mixing and decanting until the supernatant was free of nitrogenous material (5). The solid material was then placed in a kettle with a heating element and thermostat controlled at 37° C. ± 1°. Fifty-four grams of crude pancreatic powder [5] was added. Twenty-five ml. each of chloroform and toluene were added daily. This prevented any bacterial contamination as was indicated by the fact that the values for formol titration followed a smooth curve. In one experiment in which chloroform and toluene were not added daily, contamination did occur as evidenced by a foul odor and a precipitous rise in the values in the formol titration. The kettle was tightly covered and the mixture was stirred with occasional additions of pancreatic powder for a period varying from two to six weeks.

*Separation*

Frequent samples were taken and were kept in the cold room where they were ultrafiltered through a 7½ per cent collodion membrane covering an alundunum candle (45). The ultrafiltration was usually completed in the cold room after two days. The non-ultrafiltrable fraction

[5] This material and certain pancreatic digestates were supplied through the courtesy of the Armour Laboratories.

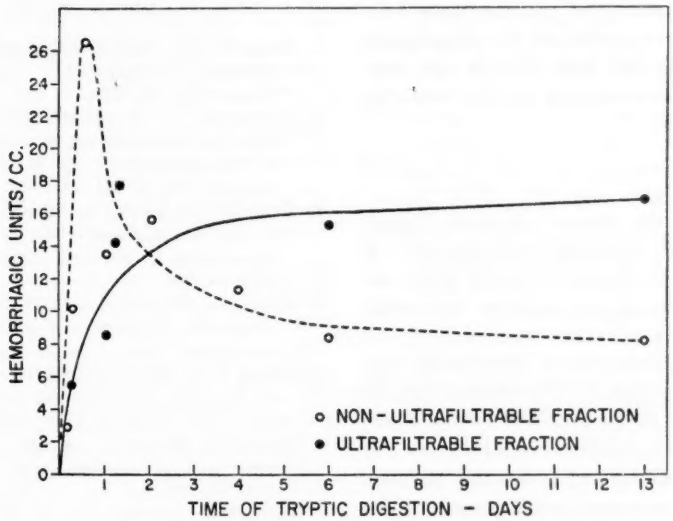


FIG. 4. THE FORMATION DURING TRYPTIC DIGESTION OF ULTRAFILTRABLE AND NON-ULTRAFILTRABLE FRACTIONS WHICH PRODUCE HEMORRHAGE ON INTRADERMAL INJECTION

The total activity of each fraction is plotted against time. Crude pancreatic powder added once at the start of digestion.

was then brought to half-saturation with ammonium sulfate to precipitate residual protein. Assays of the enzymatic activity of the supernatant (after sulfate had been removed by a second ultrafiltration) by the fibrin plate method of Astrup and Alkjaersig (46) with crystalline trypsin as the standard, indicated that less than 5 per cent of the hemorrhagic activity of the supernatant (four-hour sample) was due to non-precipitated enzyme.

Both filtrable and nonfiltrable portions were then lyophilized and used for bioassay. The appearance of toxic activity in these fractions during digestion is shown in Figure 4, in which the potency per unit volume is plotted against time. A precipitous rise in the potency of the non-ultrafiltrable material is evident which was a constant finding in numerous digestions. The potency or activity per unit weight is indicated in Table III, from which it is evident that both filtrable and nonfiltrable material retain their activity even after exhaustive digestion. The value for the non-ultrafiltrable material shows considerable variability, in excess of that expected from the error of assay. This is undoubtedly due to the errors

TABLE III

*The increase in potency of ultrafiltrable and non-filtrable fractions during pancreatic digestion*

| Time of tryptic digestion | Potency | | Formol titration* |
|---|---|---|---|
| | Non-filtrable fractions | Filtrable fractions | |
| 0 | Pancreatic powder 55 grams | | |
| | Fibrin 500 grams | | |
| 4 hours | 1.283 | | 2.01 |
| 7 hours | .914 | .1286 | 2.35 |
| 14 hours | 6.602 | | |
| 26 hours | 6.309 | .1282 | 3.22 |
| 30 hours | | .1570 | |
| 33 hours | | .1830 | |
| 49 hours | 6.815 | | |
| 4 days | 4.674 | | |
| 6 days | 8.805 | .1220 | 5.67 |
| 13 days | 4.477 | .1257 | 6.94 |
| 25 days | Pancreatic powder 27 grams | | 7.14 |
| 28 days | | | 7.20 |
| 31 days | Pancreatic powder 25 grams | | 7.38 |
| 33 days | | | 7.50 |
| 38 days | | | 7.50 |
| 40 days | Pancreatic powder 25 grams | | 7.53 |
| 41 days | 5.559 | .2021 | 7.53 |

* The completeness of digestion may be judged by the titrable acidity after addition of formaldehyde as indicated under "formol titration."

introduced in the course of treating these fractions prior to assay; nevertheless, the critical point which is evident is that both filtrable and nonfiltrable fractions retain marked activity even after exhaustive digestion.

*Discussion*

The toxicity in the skin of enzymatic digests of protein has been repeatedly demonstrated (4, 5, 9, 47, 48) although toxicity is absent in the native protein. However, no evidence has previously been available as to the time necessary for the formation and dissipation of these toxins during enzymatic digestion. The present experiments indicate that in the course of tryptic digestion, the non-ultrafiltrable fraction is progressively depleted as it is transformed to filtrable material. However, the residual nonfiltrable material gains in potency throughout digestion. The filtrable material increases both in total weight and potency during digestion.

SUMMARY

1. During pancreatic digestion of fibrin, toxic products are formed which produce local hemorrhage on intradermal injection in guinea pigs.

2. The potency and total hemorrhagic activity of the ultrafiltrable portion of the digest increases continuously until digestion is completed, *i.e.*, the ultrafiltrable toxins are not destroyed by enzymatic digestion.

3. The potency of the non-ultrafiltrable portion increases continuously until digestion is complete, although the total activity declines asymptotically.

4. An objective and quantitative method for assaying the hemorrhagic activity is described which, in a single guinea pig, yields an assay error of ± 11 per cent.

ACKNOWLEDGMENT

The author is much indebted to Professor Sven Gard of the Royal Caroline Institute, and to Professor Gunnar Olin of the State Bacteriological Laboratory, Stockholm, for providing facilities for initiating this work; to Professor Gard for his constant stimulation, help, and advice; and to Professor Frederick Fosteller of Harvard University for assistance with the statistical analysis.

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# THIAMINE AND COCARBOXYLASE CONCENTRATION IN HEART, LIVER, AND KIDNEY, OF PATIENTS WITH HEART FAILURE

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(Submitted for publication July 3, 1953; accepted July 15, 1954)

In patients with congestive heart failure, thiamine deficiency has been demonstrated by the test-dose method (1, 2), by measurement of the lactate-pyruvate ratio of the blood (3), and by determination of blood cocarboxylase (4). The oxidation of pyruvate to acetate requires thiamine in the form of cocarboxylase (thiamine pyrophosphate). Olson, Pearson, Miller, and Stare have shown that thiamine deficiency in experimental animals (rats and ducks) resulted in a decrease in the ability of cardiac muscle slices to oxidize pyruvate (5). The present investigation was thus undertaken to determine the thiamine and cocarboxylase content of cardiac muscle in patients with heart failure as compared with that of non-cardiac patients. To our knowledge no data are available on this point in the literature.

## METHODS

Patients were selected from the Philadelphia General Hospital and Temple University Hospital under treatment for congestive heart failure. In order to study the influence of long-standing heart disease upon the thiamine and cocarboxylase content of heart muscle only those patients who had failed to respond to therapy and expired with manifestations of cardiac decompensation were analyzed. There were twelve cases from whom tissues were obtained at post-mortem examination including seven with hypertensive cardiovascular disease, four with arteriosclerotic heart disease, and one with rheumatic heart disease. There were six males, and six females; their ages ranged from 39 to 81 years. The duration of treatment for heart disease in eight patients ranged from two and one-half years to eight years in the cardiac clinics or by private physicians; in four patients the duration of the disease could not be accurately determined. In each instance, treatment included the use of digitalis, mercurial diuretics or ammonium chloride, and salt restriction diets. Despite these regimens, symptoms of dyspnea, orthopnea due to pulmonary congestion, dependent edema, venous hypertension, hepatomegaly, ascites and hydrothorax (two cases) persisted, necessitating hospitalization. The patients received no supplementary vitamins but were treated with diuretic agents, digitalis, oxygen, bed rest, and restricted sodium intake. The patients expired with manifestations of advanced heart failure including Cheyne-Stokes respiration, pulmonary congestion, and general signs of anoxia. The tissues obtained for analysis of thiamine and cocarboxylase content included heart (ventricle), liver, and kidney. In the entire group, these determinations were made on wet tissues; in six cases, thiamine and cocarboxylase were measured in relation to the nitrogen content per gram of tissue.

Similar analyses were made on corresponding tissues from ten patients dying of non-cardiac causes. There were five who expired of cerebrovascular accident, one of pulmonary embolism, one of gangrene of foot, one of obstructive uropathy, one of coronary occlusion of two days' duration, and one of brain tumor. None of these patients received thiamine prior to death. Tissues from normal control subjects were unobtainable so that non-cardiac patients were used as the control group; in addition to determinations on wet tissue in each instance, analysis was made for thiamine and cocarboxylase content based on the dry weight and compared to the nitrogen content per gram of tissue in six non-cardiac cases.

Within one hour after death, the body was placed in an ice box at temperature of 38° F. and within five hours after death tissues were removed, kept frozen at minus 10° F. until immediately before they were analyzed. Pieces of tissue weighing about 100 grams were dissected from tendon and fat. A sample of tissue weighing approximately 50 grams was homogenized in distilled water (about 1 weight tissue and 2 weight water) using a Waring Blender. Analysis was made of this homogenate for free thiamine using a modified method of Hochberg and Melnick (6). The mixture was permitted to stand for one hour at room temperature and filtered through No. 40 Whatman filter paper. Sodium acetate 2 N was added to the mixture to adjust the pH to 4.5. It was passed through Zeolite (Decalso column) filter tube. Boiling distilled water was passed through Decalso column and free thiamine was determined utilizing the reaction between thiamine and p-amino-acetophenone. A second sample of tissue was used for analysis of total thiamine and cocarboxylase, using a modification of the method of Hochberg, Melnick, and Oser (7). Approximately 50 grams of the tissue were macerated in a Waring Blender with 0.1 N $H_2SO_4$, and the volume was brought to 250 ml. with 0.1 N $H_2SO_4$. The mixture was digested for one hour at 100° C.; and then cooled to room temperature. The mixture was adjusted to the pH of 4.5 with 2 N sodium acetate. Clarase®,[1] 3 per cent by

[1] We are indebted to Takamine Laboratory for the supply of Clarase®.

weight of tissue, was added and incubated at 37° for 12 hours. This completed the hydrolysis of cocarboxylase to thiamine. It was filtered and aliquot parts (100 ml.) passed through the Zeolite (Decalso column) filter and thiamine was determined (7). The cocarboxylase was calculated by the difference between free thiamine of the first homogenate and the total thiamine of the second homogenate. Total nitrogen was determined by the micro-Kjeldahl method.

## RESULTS

The analyses of heart, liver, and kidney tissues for total thiamine and cocarboxylase are presented in Tables I and II. These data express the values obtained as micrograms of these substances per gram of the wet weight of tissues. The mean values for the cardiac cases were for thiamine 0.60 microgram per gram of tissue and for cocarboxylase 0.36 microgram per gram of tissue; in the non-cardiac cases, the mean values for thiamine were 1.37 micrograms per gram of tissue and for cocarboxylase 0.84 micrograms per gram of tissue.

The difference between the means of total thiamine and cocarboxylase in patients with car-

TABLE I

*Cardiac patients—concentration of total thiamine and cocarboxylase in heart, liver, and kidney* *

| Patient | Age | Sex | | Heart | Liver | Kidney | Remarks |
|---|---|---|---|---|---|---|---|
| A. O. | 60 | F | Th | 0.52 | 0.54 | 0.87 | Hypertensive cardiovascular disease. Cardiac failure. Died on 5th hospital day. |
| | | | Coc. | 0.34 | 0.48 | 0.71 | |
| R. S. | 66 | F | Th | 0.63 | 0.85 | 0.81 | Arteriosclerotic heart disease. Residual left hemiplegia. Cardiac failure. Died on 17th hospital day. |
| | | | Coc. | 0.50 | 0.75 | 0.68 | |
| A. C. | 81 | F | Th | 0.88 | 0.27 | 0.71 | Hypertensive cardiovascular disease. Thrombophlebitis—right lower extremity. Cardiac failure. Died on 35th hospital day. |
| | | | Coc. | 0.59 | 0.23 | 0.60 | |
| J. T. | 70 | M | Th | 0.89 | 1.07 | 0.77 | Arteriosclerotic heart disease. Apical myocardial infarct; myocardial failure. Died on 10th hospital day. |
| | | | Coc. | 0.70 | 0.84 | 0.59 | |
| S. B. | 39 | F | Th | 0.86 | 0.75 | 1.9 | Malignant hypertension. Cardiac failure. Died on 14th hospital day. |
| | | | Coc. | 0.45 | 0.56 | 1.49 | |
| W. R. | 80 | M | Th | 0.43 | 0.41 | 1.03 | Hypertensive cardiovascular disease. Pulmonary edema. Died after 6 hours hospitalization. |
| | | | Coc. | 0.33 | 0.23 | 0.77 | |
| F. C.† | 63 | M | Th | 0.81 | 0.33 | 0.53 | Hypertensive cardiovascular disease. Cardiac failure. Died on the 12th hospital day. |
| | | | Coc. | 0.52 | 0.28 | 0.45 | |
| K. H.† | 70 | F | Th | 0.29 | 0.52 | 1.10 | Arteriosclerotic heart disease. Congestive failure. Died on the 3rd hospital day. |
| | | | Coc. | 0.06 | 0.30 | 0.78 | |
| P. F.† | 66 | F | Th | 0.76 | 0.38 | 0.67 | Hypertensive cardiovascular disease. Died on 3rd hospital day. |
| | | | Coc. | — | 0.27 | 0.51 | |
| J. C.† | 78 | M | Th | 0.69 | 0.40 | 2.32 | Arteriosclerotic heart disease. Died on the 11th hospital day. |
| | | | Coc. | 0.23 | 0.16 | 1.26 | |
| M. F.† | 77 | M | Th | 0.39 | 0.41 | 0.66 | Hypertensive cardiovascular disease. Cardiac failure. Died on the 1st hospital day. |
| | | | Coc. | 0.23 | 0.34 | 0.54 | |
| R. M.† | 73 | M | Th | 0.02 | 0.16 | 0.16 | Rheumatic heart disease. Congestive failure. Died on 6th hospital day. Three previous admissions for cardiac failure. |
| | | | Coc. | 0 | 0.16 | 0.16 | |
| Means of tissue | | | Th | 0.60 | 0.51 | 0.96 | |
| | | | Coc. | 0.36 | 0.38 | 0.71 | |

* Results are expressed in micrograms per gram of wet tissue.
† Measurements are also available per gram of tissue nitrogen.

TABLE II

*Non-cardiac patients—concentration of total thiamine and cocarboxylase in heart, liver, and kidney* *

| Patient | Age | Sex | | Heart | Liver | Kidney | Remarks |
|---|---|---|---|---|---|---|---|
| D. K. | 70 | F | Th | 1.43 | 1.35 | 1.45 | Hypertension. Cerebral hemorrhage. Died on the 14th hospital day. |
| | | | Coc. | 1.16 | 0.94 | 0.54 | |
| M. S. | 74 | F | Th | 1.2 | 1.7 | 1.2 | Cerebral thrombosis. Bronchopneumonia. Died on the 8th hospital day. |
| | | | Coc. | 0.82 | 1.43 | 0.82 | |
| E. H. | 52 | M | Th | 0.99 | 0.89 | 1.1 | Pulmonary embolism; drug addiction; diverticulosis; transverse colostomy. Died on the 18th hospital day. |
| | | | Coc. | 0.81 | 0.71 | 0.85 | |
| M. S. | 65 | F | Th | 1.21 | 1.08 | 2.26 | Cerebral hemorrhage due to hypertension. Died on the 11th hospital day. |
| | | | Coc. | 0.69 | 0.57 | 1.40 | |
| M. H.† | 57 | F | Th | 1.67 | 0.95 | 0.79 | Cerebrovascular accident. Died on the 10th hospital day. |
| | | | Coc. | 0.55 | 0.60 | 0.30 | |
| I. P.† | 75 | M | Th | 1.39 | 0.79 | 1.46 | Amputation of left leg for gangrene. Died on the 3rd hospital day. |
| | | | Coc. | 0.85 | 0.48 | 1.18 | |
| E. G.† | 69 | M | Th | 1.27 | 0.72 | 1.27 | Cerebral hemorrhage. Died on the 3rd hospital day. |
| | | | Coc. | 0.52 | 0.16 | 0.52 | |
| T. J.† | 62 | M | Th | 1.62 | 0.95 | 1.20 | Obstructive uropathy. Bronchopneumonia. Died on the 5th hospital day. |
| | | | Coc. | 1.47 | 0.64 | 0.80 | |
| T. C.† | 59 | F | Th | 1.88 | 1.04 | 1.87 | Coronary occlusion. No previous history of heart disease. Died on the 2nd hospital day. |
| | | | Coc. | 0.90 | 0.81 | 0.80 | |
| S. M.† | 58 | F | Th | 1.01 | 0.31 | 0.99 | Brain tumor. Died on the 5th postoperative day. |
| | | | Coc. | 0.58 | 0.10 | 0.59 | |
| Means of tissue | | | Th | 1.37 | 0.98 | 1.36 | |
| | | | Coc. | 0.84 | 0.64 | 0.78 | |

* Results are expressed in micrograms per gram of wet tissue.
† Measurements are also available per gram of tissue nitrogen.

TABLE III

*Statistical evaluation of total thiamine and cocarboxylase in wet tissues of twelve* cardiac and ten non-cardiac patients*

| | Heart | | Liver | | Kidney | |
|---|---|---|---|---|---|---|
| | Thiamine *Micrograms per gram* | Cocarbox. *Micrograms per gram* | Thiamine *Micrograms per gram* | Cocarbox. *Micrograms per gram* | Thiamine *Micrograms per gram* | Cocarbox. *Micrograms per gram* |
| Cardiac cases | | | | | | |
| Mean | 0.60 | 0.36 | 0.51 | 0.38 | 0.96 | 0.71 |
| Standard deviation | 0.26 | 0.21 | 0.25 | 0.22 | 0.57 | 0.34 |
| Control cases | | | | | | |
| Mean | 1.37 | 0.84 | 0.98 | 0.64 | 1.36 | 0.78 |
| Standard deviation | 0.27 | 0.28 | 0.35 | 0.36 | 0.41 | 0.31 |
| Mean of control cases minus mean of cardiac cases | +0.77 | +0.48 | +0.47 | +0.26 | +0.40 | +0.07 |
| Probability of "t" for difference between means | Less than .001 | Less than .001 | Less than .01 | .06 | .1 | .6 |
| | Highly significant | Highly significant | Highly significant | Non-significant | Non-significant | Non-significant |

* Data for cocarboxylase in heart missing from one cardiac case.

diac failure and the means in the control patients was tested by means of the "t" test. The probabilities of the "t" are listed in Table III. The conventional 0.05 level of significance was used. It is apparent that in the case of the heart tissue, there was a highly significant difference between the two groups of patients both for total thiamine and cocarboxylase ($P < .001$). In the liver tissue, the difference between the two groups was highly significant for total thiamine but not significant for cocarboxylase. In the kidney tissue from the control group both thiamine and cocarboxylase were higher than in the kidney tissues from the cardiac group. The difference between the means, however, was statistically not significant.

In six of the cardiac patients and in six control patients, the total thiamine and cocarboxylase expressed in micrograms per gram of tissue nitrogen are presented in Tables IV and V. The mean values for the cardiac patients for thiamine were 17.25 micrograms per gram of nitrogen of heart tissue, and for cocarboxylase 7.14 micrograms per gram of nitrogen of heart muscle, and in the non-cardiac cases 56.7 micrograms of thiamine per gram of nitrogen of heart muscle and 32.03 micrograms of cocarboxylase per gram of heart tissue nitrogen. The statistical evaluation of the total thiamine and cocarboxylase per gram of tissue nitrogen in both cardiac and non-cardiac cases is presented in Table VI. It is evident that the results are similar in the ashed tissues to those obtained using the wet tissues.

## DISCUSSION

The results of this study tend to indicate that all three types of tissues had a lower concentration of both total thiamine and cocarboxylase in patients with cardiac failure as compared with those who died of other causes. The differences, however, were statistically significant for total thiamine and cocarboxylase in heart only and for total thiamine alone in liver tissue.

The results are in agreement with experimental data reported by several investigators that dietary restriction of thiamine results in a decrease of tissue concentration of both thiamine and cocarboxylase (8, 9). We and others have suggested that patients with heart failure are apt to show thiamine deficiency because of inadequate intake of thiamine in the food, poor absorption of ingested thiamine, and also as a result of the use of mercurials for elimination of edema (2, 3, 10).

TABLE IV

*Concentration of total thiamine and cocarboxylase in tissues from six cardiac patients**

| Name | Age | Sex | | Heart | Liver | Kidney | |
|---|---|---|---|---|---|---|---|
| F. C. | 63 | M | Thiamine | 26.9 | 11 | 20.1 | Hypertensive cardiovascular disease. Cardiac failure. Died on the 12th hospital day. |
| | | | Cocarbox. | 17.3 | 9.3 | 17.1 | |
| K. H. | 70 | F | Thiamine | 9.9 | 15.8 | 43.7 | Congestive heart failure. Arteriosclerotic heart disease. Died on the 3rd hospital day. |
| | | | Cocarbox. | 2.0 | 9.4 | 31.0 | |
| P. F. | 66 | F | Thiamine | 28.3 | 12.5 | 31.3 | Hypertensive cardiovascular disease. Cardiac failure. Died on the 3rd hospital day. |
| | | | Cocarbox. | — | 8.9 | 23.9 | |
| J. C. | 78 | M | Thiamine | 22.5 | 12.08 | 78.65 | Arteriosclerotic heart disease. Died on the 11th hospital day. |
| | | | Cocarbox. | 7.52 | 5.17 | 42.72 | |
| M. F. | 77 | M | Thiamine | 15.02 | 14.02 | 30.29 | Hypertension. Cardiac failure. Died after 14 hours hospitalization. |
| | | | Cocarbox. | 8.86 | 11.63 | 24.78 | |
| R. M. | 73 | M | Thiamine | 0.88 | 5.17 | 7.62 | Rheumatic heart disease. Cardiac failure. Three previous admissions. Died on the 6th hospital day. |
| | | | Cocarbox. | 0 | 5.11 | 7.62 | |
| Means of tissue | | | Thiamine | 17.25 | 11.76 | 35.28 | |
| | | | Cocarbox. | 7.14 | 8.25 | 24.52 | |

* The results are expressed in micrograms per gram of tissue nitrogen.

TABLE V

*Concentration of total thiamine and cocarboxylase in tissues from six non-cardiac patients**

| Name | Age | Sex | | Heart | Liver | Kidney | |
|---|---|---|---|---|---|---|---|
| M. H. | 57 | F | Thiamine | 76.0 | 66.4 | 58.5 | Cerebrovascular accident. Died on 10th hospital day. |
| | | | Cocarbox. | 25.4 | 41.9 | 22.2 | |
| I. P. | 75 | M | Thiamine | 53.8 | 30.5 | 70.2 | Amputation of left foot for gangrene. Died on 3rd hospital day. |
| | | | Cocarbox. | 32.9 | 18.5 | 56.8 | |
| E. G. | 69 | M | Thiamine | 50.4 | 30.1 | 50.4 | Cerebral hemorrhage. Died on 3rd hospital day. |
| | | | Cocarbox. | 31.4 | 23.4 | 31.4 | |
| T. J. | 62 | M | Thiamine | 51.35 | 25.18 | 51.48 | Obstructive uropathy. Bronchopneumonia. Died on 5th hospital day. |
| | | | Cocarbox. | 46.59 | 16.96 | 35.32 | |
| T. C. | 59 | F | Thiamine | 70.50 | 36.19 | 72.74 | Coronary occlusion. Died after 48 hours' hospitalization. No previous history of heart disease. |
| | | | Cocarbox. | 33.75 | 28.18 | 31.11 | |
| S. M. | 58 | F | Thiamine | 38.58 | 10.17 | 34.16 | Brain tumor. Died on 5th postoperative day. |
| | | | Cocarbox. | 22.15 | 2.95 | 20.36 | |
| Means of tissue | | | Thiamine | 56.77 | 33.09 | 56.25 | |
| | | | Cocarbox. | 32.03 | 21.98 | 32.87 | |

* The results are expressed in micrograms per gram of tissue nitrogen.

Our analyses were done on tissues obtained at the post-mortem table. The results of the total thiamine are probably valid because of the fact that post-mortem autolysis has an insignificant effect upon thiamine content of tissues according to Ferrebee, Weissman, Parker, and Owen (11). The evaluation of cocarboxylase in our post-mortem material is difficult. Apparently no data are available on the stability of thiamine pyrophosphate under these conditions but analyses in both cardiac and control cases on tissues obtained under similar conditions permit a meaningful comparison. In this connection the findings of Ferrebee, Weissman, Parker, and Owen (11) are of interest. In their analysis of the thiamine content of heart and liver tissues obtained from per-

TABLE VI

*Statistical evaluation of the total thiamine and cocarboxylase in tissues of six cardiac and six non-cardiac patients**

| | Heart | | Liver | | Kidney | |
|---|---|---|---|---|---|---|
| | Thiamine | Cocarbox. | Thiamine | Cocarbox. | Thiamine | Cocarbox. |
| Cardiac cases | | | | | | |
| Mean | 17.25 | 7.14† | 11.76 | 8.25 | 35.28 | 24.52 |
| Standard deviation | 7.94 | 6.06 | 3.32 | 2.37 | 22.32 | 10.91 |
| Control cases | | | | | | |
| Mean | 56.77 | 32.03 | 33.09 | 21.98 | 56.25 | 32.87 |
| Standard deviation | 12.70 | 7.72 | 16.94 | 11.81 | 13.02 | 11.93 |
| Mean of control cases minus mean of cardiac cases | +39.52 | +24.89 | +21.33 | +13.73 | +20.97 | + 8.35 |
| Probability of "t" for difference between means | Less than .001 | Less than .001 | .04‡ | .05+‡ | .1 | .3 |
| | Highly significant | Highly significant | Significant | Non-significant | Non-significant | Non-significant |

* The mean values are expressed as micrograms per gram of tissue nitrogen.
† Data for cocarboxylase in heart tissue were missing for one cardiac case.
‡ Adjusted for unequal variances.

sons in poor nutritional status with respect to this vitamin, values were found which correspond closely to those obtained in heart and liver tissues of our patients who died of heart failure.

On the other hand, total thiamine in heart tissue of autopsy material from three subjects who met accidental death ranged between 2.5 to 4.5 micrograms per gram of tissue (average 3.7 micrograms per gram) (12). These values are considerably higher than were found in our control cases. A possible explanation for this is the better state of nutrition of subjects who met accidental death.

Whether the thiamine content of heart muscle in the cardiac cases was at such a low level as to impair the utilization of pyruvate we are unable to state. In several studies of the respiration and utilization of substrate by slices of cardiac muscle from experimental animals it was shown that large decreases of heart muscle thiamine were required before impairment of pyruvate utilization occurred but below 2.5 micrograms of thiamine per gram fresh weight of cardiac muscle the conversion of pyruvate to non-lactate products was a function of the thiamine content (13). Whether a similar metabolic behavior obtains in human heart is not established. Manifestations of thiamine deficiency were not observed in these patients with the exception of one who had malnutrition, smooth red tongue, and hyporeflexia (Case R. M.). It is well known that tissue thiamine levels at which symptoms of thiamine deficiency develop vary with the amount of physiologic activity but it is reasonable to assume that the low total thiamine and cocarboxylase content of the myocardium had a deleterious effect on cardiac muscle metabolism. Whether this factor played a role in the refractoriness of patients to cardiac therapy is a point for further study.

The liver tissues from the cardiac cases showed a significantly lower total thiamine content than from the control cases. This may be explained by the fact that in cardiac failure liver damage is of frequent occurrence (14) and as Borson (15) has suggested hepatic dysfunction may lead to a decreased phosphorylation of thiamine with a consequent elimination of the vitamin in the urine.

In attempting to explain the small difference between thiamine and cocarboxylase content of the kidney tissue in the groups of patients, we must bear in mind that in both groups the thiamine stores were below normal. In thiamine depletion the low tissue thiamine concentration may increase the rate of recovery of thiamine from urine in order to replete body stores of this vitamin (16). Such a process would be expected to increase the thiamine content of renal tissue.

## SUMMARY

Heart, liver, and kidney tissues were analyzed for thiamine and cocarboxylase content in 12 cardiac and 10 non-cardiac patients. The analyses were made in wet tissues in the entire series and in six of each group the results were also related to the nitrogen content of the tissues.

In all tissues examined, there were lower concentrations of both total thiamine and cocarboxylase in patients with cardiac failure than in patients who died of other causes. The differences were statistically significant for both total thiamine and cocarboxylase in heart tissue and for total thiamine in liver tissue.

The finding of the significantly reduced thiamine and cocarboxylase contents of heart muscle is worthy of further investigation as to its possible influence upon myocardial metabolism in heart failure.

## ACKNOWLEDGMENTS

We are indebted to Doctors Henry Swartz and James Hazlett Jones for supervision and constant advice on work in progress; to Dr. H. Menduke for statistical treatment and to Dr. William E. Ehrich for permission to use autopsy material.

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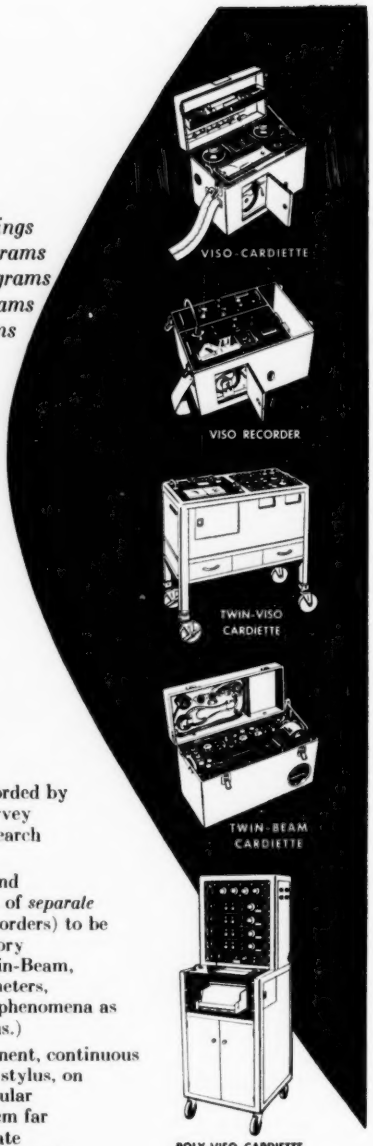


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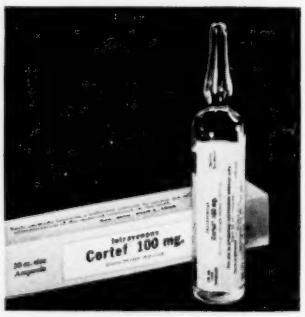

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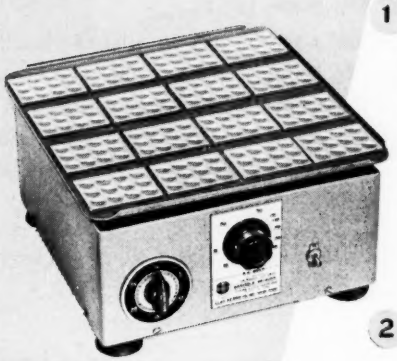

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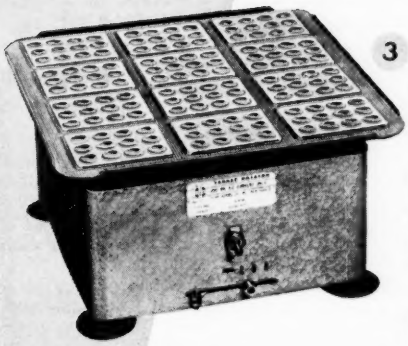
- Direct setting of speeds by dial
- Adjustable from 60-270 RPM
- Automatic timer—can be set from 1-30 minutes
- Precise heavy-duty construction

**A-2273** Yankee AC Variable Speed Rotator, 13 x 13" platform, speed variable from 60-270 RPM, automatic timer from 1-30 minutes for 110-220 V, 60 cycle AC... **each $127.50**

## 2 Yankee AC-DC Variable Speed Rotator

Similar in appearance to A-2273 above. Speed settings controlled by rheostat. Where voltage and load are constant, direct calibration of the rheostat is possible.

**A-2272** Yankee AC-DC Variable Speed Rotator, 13 x 13" platform, speeds variable from approximately 70-210 RPM, automatic timer from 1-30 minutes, for 110-120 volts AC-DC... **each $90.00**



## 3 Yankee Rotators

- Automatic timer, or continuous operation
- For greater uniformity and control of routine procedures
- Two models: 130 RPM and 180 RPM

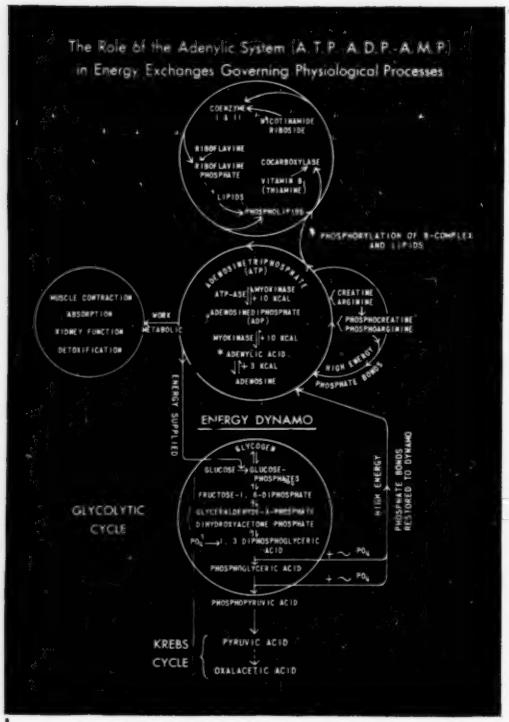
**A-2270**—130 RPM, for blood typing and cross-matching. 11 x 11" platform with automatic timer, for 110-120 V, 60 cycle AC... **each $69.00**

**A-2271**—180 RPM for VDRL, Klein and Rein-Bossak tests. 11 x 11" platform with automatic timer for 110-120 V, 60 cycle AC... **each $69.00**

All Yankee Rotators describe a ¾" diameter circle; all are rubber suction cup-mounted to prevent creeping.

***Order From Your Local Supply Dealer***

**Clay-Adams** **141 E. 25th Street, New York 10, N. Y.**

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Gradually the role of the Adenylic Nucleotides is being clarified. Their versatility and importance both physiologically and in clinical medicine is leading to applications in an expanding number of clinical indications.*

We have available the following adenylic nucleotides in one cc ampuls:

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**IRON ADENOSINE-3-MONOPHOSPHATE**
Each one cc ampul contains 32.4 mg. of Ferrous Adenylate equivalent to 28 mg. of adenosine-3-monophosphate and 4.4 mg. of ferrous iron.

**Available in boxes of 10, 25 and 100 ampuls**

**MUSANYL®**

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Each one cc ampul contains 32.4 mg. of Ferrous Adenylate equivalent to 28 mg. of adenosine-5-monophosphate and 4.4 mg. of ferrous iron.

**Ampuls in boxes of 10s, 25s and 100s**

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**Available in bottles of 12, 25 and 250 tablets**

## AVAILABLE ONLY TO THE CLINICAL INVESTIGATOR:

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**NICOTINAMIDE ADENYLATE** — NICOTINAMIDE ADENOSINE-3-MONOPHOSPHATE. Each one cc ampul contains 52 mg. of Nicotinamide Adenylate equivalent to 22 mg. of nicotinamide and 30 mg. of adenosine-3-monophosphate.

**NIKETHAMIDE ADENYLATE** — NIKETHAMIDE ADENOSINE-3-MONOPHOSPHATE. Each one cc ampul contains 80 mg. of Nikethamide Adenylate equivalent to 50 mg. of Nikethamide and 30 mg. of adenosine-3-monophosphate.

**NATRI-A. T. P.** — SODIUM ADENOSINE-5-TRIPHOSPHATE. Each one cc ampul contains 10.67 mg. of Sodium Adenosine-5-Triphosphate equivalent to 9 mg. of Adenosine-5-Triphosphate and 1.67 mg. of sodium.

*An annotated Bibliography, "ABSTRACTS ON THE ADENYLIC NUCLEOTIDES with particular reference to Yeast Adenylic Acid (Adenosine-3-Monophosphate) is available on request.

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# CLINICAL REPORT ON ANSOLYSEN . . .
# NEW ANTIHYPERTENSIVE AGENT

ANSOLYSEN—pentolinium tartrate—is a potent ganglionic blocking agent which presents the advantage of comparative freedom from by-effects. This effective hypotensive agent is recommended for use in patients with moderate to severe hypertension.

Comparing the effects of hexamethonium and ANSOLYSEN in 27 patients with severe "fixed" hypertension, Freis and coworkers[1] observed:

ANSOLYSEN was approximately five times more potent than hexamethonium

ANSOLYSEN produced less tolerance

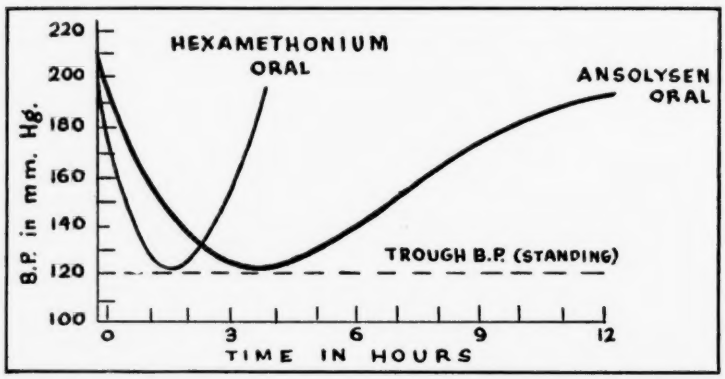
ANSOLYSEN's hypotensive effect was 40% longer

ANSOLYSEN's hypotensive effect was more predictable

ANSOLYSEN caused less pronounced by-effects

ANSOLYSEN caused less constipation

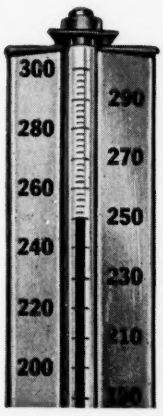
ANSOLYSEN lowered the blood pressure significantly, with little or no risk of producing collapse reactions or paralytic ileus




Changes in blood pressure noted by Smirk[2] after therapeutic oral doses of hexamethonium and ANSOLYSEN.

Supplied: Scored Tablets—40 and 100 mg., bottles of 100
Injection—10 mg. per cc., vials of 10 cc.


1. Freis, E. D., and others: Circulation 9:540 (April) 1954
2. Smirk, F. H.: New Zealand M.J. 52:1 (Oct.) 1953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